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Orientadores: Professor Doutor Frederico Simões do Couto
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Dissertação especialmente elaborada para obtenção do grau de Mestre em Neurociências

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Abstract

Introduction: Over the last 30 years, use of Transcranial Magnetic Stimulation (TMS) has grown exponentially, from therapeutic application in major depressive disorder (MDD) to research use for modulation of cortical activity regions or assessment of neuronal excitability. TMS modulatory interventions, through repetitive Transcranial Magnetic Stimulation (rTMS), have been regularly associated with the manipulation of neuroplasticity-like phenomena (Hallet *et al*, 2007). Nonetheless, assessing these effects *in vivo* in human models is challenging. Some studies have demonstrated this phenomenon using Electromyography (EMG), through measurement of Motor Evoked Potential (MEP) amplitude change after rTMS of the Primary Motor Cortex (M1) (e.g. Maeda *et al*, 2000). Furthermore, this physiological marker (ΔMEP) has shown predictive properties for rTMS treatment-response in patients with MDD (Oliveira-Maia *et al*, 2017).

Objectives: My main aim was to confirm the modulatory effects of 10Hz rTMS applied on the left M1, on corticospinal excitability of the contralateral upper limb. Exploratory analyses of contralateral effects on corticospinal excitability, potentially modulated by inter-hemispheric modulation of the non-stimulated motor cortex, were also performed. Finally, I explored variables that influenced excitability modulation in order to understand potential improvements of the data acquisition protocol.

Methods: After confirmation of eligibility and psychometric assessment, TMS was performed. Initially, Motor Threshold (MT) and MEPs were acquired from both the left and right hemisphere. After 10 Hz rTMS of the left M1, MEPs were re-assessed and variation of MEP amplitude from pre- to post-rTMS (ΔMEP) was calculated for both hemispheres, as a measure of excitability modulation.

Results: Thirty-two healthy volunteers were enrolled. Significant modulatory effects of rTMS were found on the left hemisphere ($p = 0.032$), in accordance with previous studies (e.g. Maeda *et al* 2000). No effects were found contralaterally. Regarding the impact of several factors concerning experimental design, I found that participants in whom MEPs were assessed first in the left M1 demonstrated a robust modulatory effect ($p = 0.046$), which is not observed in participants who were assessed first in the right hemisphere (*p*

= 0.5). Furthermore, modulatory effects were conserved in participants in whom TMS was performed in the morning (p = 0.04), but not those tested in the afternoon (p = 0.6). Further exploratory analyses, comparing participants with and without prior history of MDD showed no differences (p = 0.9).

Conclusion: The present study confirms an increase in MEP mean amplitude after 10Hz rTMS delivered to the left M1 protocol, and the absence of modulatory effects of the contralateral hemisphere. The order by which hemispheres are assessed and the moment of the day the TMS session takes place seem to affect the modulatory effect, with enhanced modulation in participants starting MEP assessment in the left hemisphere or participants assessed in the morning. Absence of differences between participants with previous depressive episodes suggest that any potential depression-related differences in neuroplastic processes, may be transient state-markers, rather than trait-markers. Nevertheless, this data contributes for definition of adequate protocol for *in vivo* comparisons of excitability modulation between patients with MDD and healthy volunteers.

Keywords: Transcranial magnetic stimulation, Corticospinal excitability, Neuroplasticity, ΔMEP, Major depressive disorder

Resumo

Introdução: Ao longo dos últimos 30 anos, o uso da Estimulação Magnética Transcraniana (EMT) tem crescido exponencialmente, desde a sua aplicação terapêutica na perturbação depressiva major (PDM), até à utilização na investigação da modulação da atividade de regiões corticais ou a avaliação de excitabilidade neuronal. EMT é uma técnica que se baseia na indução eletromagnética. Através da indução de uma corrente elétrica numa bobine, são gerados campos eletromagnéticos perpendiculares a esta que, por sua vez, ao interagir com um material condutor, por exemplo, o tecido cerebral, geram a corrente elétrica nesse material. Com base neste mecanismo, podem considerar-se duas formas de aplicação da técnica. A primeira é designada por EMT de pulsos únicos, que podem ser aplicados, por exemplo, na região do córtex motor primário (M1), responsável pela atividade de um determinado músculo da mão. Desta forma, estes pulsos podem gerar uma resposta fisiológica, registada através de eletromiografia (EMG), sendo esta considerada uma medida de excitabilidade cortical, i.e, uma medida da ativação das populações neuronais subjacentes à estimulação. A segunda forma da EMT a ser considerada está relacionada com a aplicação de pulsos repetidos de acordo com determinados protocolos, em que o fator frequência parece ser determinante na tendência do efeito modulatório deste mecanismo. Assim, altas frequências (≥5Hz) tendem a facilitar a excitabilidade cortical da região estimulada para além do período de estimulação propriamente dita, sendo que o inverso acontece com frequências mais reduzidas (≤1Hz) que inibem a excitabilidade (Fitzgerald *et al*, 2004). Esta abordagem designa-se por EMT repetitivo (EMTr).

Diversas aplicações destas abordagens têm surgido para o estudo do cérebro humano *in vivo*, tanto em contexto normativo, como patológico. Particularmente, o estudo de efeitos modulatórios da excitabilidade cortical torna-se possível através do emparelhamento de EMT com EMG, onde as medições de excitabilidade cortical, através de Potenciais Evocados Motores (PEM), são feitas antes e após um protocolo de EMTr. Estas são designadas por medidas de modulação da excitabilidade cortical. Um dos grandes interesses destas medidas prende-se com a sua associação à manipulação de fenómenos de neuroplasticidade (Hallet *et al*, 2007), cujo estudo *in vivo* é desafiante em humanos. Na verdade, a avaliação de processos de neuroplasticidade é muito relevante em contexto clínico, particularmente no que diz respeito a perturbações neuropsiquiátricas como a

PDM. Na verdade, uma das teorias que propõe explicar a fisiopatologia da doença sugere que os doentes com depressão apresentam défices em termos de processos neuroplásticos (Feldman, 2009). Assim, importa perceber se os valores obtidos em medidas de modulação da excitabilidade cortical, em doentes com depressão, se encontram de facto disfuncionais, em comparação com sujeitos saudáveis. Tal iria confirmar a presença de défices de processos plásticos em contexto desta doença, podendo mesmo permitir o desenvolvimento de um marcador de diagnóstico.

Num dos estudos de EMTr do córtex motor foi demonstrado que a aplicação de um protocolo de 10Hz no córtex motor esquerdo induz facilitação da excitabilidade cortical em voluntários saudáveis (Maeda *et al*, 2000). Por outro lado, num outro estudo, esta medida demonstrou ter propriedades preditivas da resposta a EMTr para tratamento de depressão (Oliveira-Maia *et al*, 2017). Ou seja, antes de tratamento com um ciclo de EMTr prefrontal, foi aferida, em doentes com depressão esta medida de modulação da excitabilidade do córtex motor. Os resultados demonstraram que, quanto maior o valor obtido nesta medida, maior a resposta ao tratamento (Oliveira-Maia *et al*, 2017). Neste estudo foram também comparados, indiretamente, os valores médios da medida referida com aqueles obtidos em voluntários saudáveis no estudo de Maeda e colaboradores (2000), verificando-se uma diferença limítrofe com os resultados obtidos em doentes com depressão. Naturalmente, estes resultados carecem de corroboração por comparação direta entre as duas populações, no que diz respeito a esta medida de modulação da excitabilidade cortical.

Por fim, alguma evidência sugere que os efeitos modulatórios de EMTr num determinado hemisfério cerebral são acompanhados de efeitos de sentido contrário (facilitação/inibição) na zona correspondente do hemisfério contralateral. Assim, Bajwa e colaboradores (2008) verificaram que, após um protocolo modulatório de baixa frequência (1Hz), a excitabilidade cortical do hemisfério ipsilateral à estimulação reduzia, havendo uma facilitação da desta no M1 contralateral. No entanto, a evidência a suportar este achado é limitada, pelo que, mais estudos são necessários para a sua sustentação.

Objetivos: O meu objetivo principal foi confirmar o efeito modulatório de EMTr de 10Hz administrado no M1 esquerdo, na excitabilidade corticoespinhal do membro superior contralateral, mais concretamente, no primeiro músculo interósseo dorsal da mão (FDI).

Foram igualmente realizadas análises exploratórias de efeitos contralaterais na excitabilidade cortical, potencialmente através de modulação interhemisférica do córtex motor não estimulado. Por fim, por forma a explorar potenciais melhorias do protocolo de aquisição de dados, analisei diversas variáveis que influenciaram a modulação da excitabilidade.

Métodos: Uma vez que se pretendia o recrutamento de pessoas saudáveis, o primeiro passo da sessão passou pela confirmação de elegibilidade com especial incidência no diagnóstico de doença neuropsiquiátrica, através da Structured Clinical Interview for DSM-IV (SCID), para PDM e da Mini International Neurospychiatric Inventory (MINI) para outros diagnósticos. Posteriormente, foi feita avaliação psicométrica. De seguida, procedeu-se à sessão de EMT. Inicialmente foram adquiridos o Limiar Motor (LM) e PEMs em ambos os hemisférios, sendo administrados 31 pulsos em cada hemisfério. A ordem pela qual os hemisférios foram avaliados foi randomizada antes das recolhas serem iniciadas. De seguida, foi aplicado um protocolo EMTr de 10Hz no M1 esquerdo e, finalmente os PEM's foram reavaliados. A variação da sua amplitude do pré para o pós-protocolo de EMTr (ΔMEP) foi calculada para ambos os hemisférios, traduzindo uma medida de modulação de excitabilidade, i.e., um *proxy* de neuroplasticidade para cada um dos indivíduos.

Resultados: Foram recrutados para o estudo 32 sujeitos saudáveis. Tal como previsto, os resultados obtidos sugerem a existência de um efeito modulatório significativo no hemisfério esquerdo ($p = 0.032$), tal como em estudos anteriores (e.g. Maeda *et al*, 2000). No entanto, contrariamente ao esperado em termos de efeitos contralaterais da modulação, nenhuma mudança foi encontrada no hemisfério não estimulado. No que diz respeito ao impacto de diversos fatores referentes ao desenho experimental, verificámos que participantes a quem o M1 esquerdo foi avaliado em primeiro lugar, demonstraram um efeito modulatório robusto ($p = 0.046$), algo que não é observado nos participantes em que a avaliação começou no hemisfério direito ($p = 0.5$). Para além do mais, os efeitos modulatórios mantiveram-se conservados em participantes cuja sessão de EMT ocorreu de manhã ($p = 0.04$), mas não naqueles testados durante a tarde ($p = 0.6$). Outras análises exploratórias, que compararam participantes com e sem história prévia de PDM não revelaram diferenças ($p = 0.9$).

Conclusão: Este estudo confirma um aumento na amplitude média de PEMs após um protocolo de EMTr de 10Hz administrado no M1 esquerdo e a ausência de efeitos modulatórios no hemisfério contralateral, contrariando resultados obtidos em estudos prévios (e.g. Bajwa *et al*, 2008). A ordem pela qual os hemisférios são avaliados parece ter uma influência considerável nos efeitos modulatórios, algo raramente reportado em estudos que têm como objetivo a avaliação das relações interhemisféricas usando EMT, podendo explicar as diferenças obtidas entre os nossos resultados e os presentes em trabalhos anteriores. Para além do mais, o momento do dia em que a sessão de EMT ocorre aparenta também afetar o efeito modulatório, com aumento deste em participantes que começaram a avaliação de PEMs à esquerda ou participantes avaliados de manhã, podendo indicar uma diferente sensibilidade a EMT em função do momento do ciclo sono-vigília, como sugerido em estudos prévios (Cohen *et al*, 2010). A semelhança dos dados de participantes com episódios depressivos prévios sugere que eventuais diferenças ao nível dos mecanismos de neuroplasticidade associadas à depressão sejam marcadores de estado e não de traço. Estes dados contribuem para a definição de um protocolo adequado para comparações *in vivo* de modulação de excitabilidade entre doentes com PDM e voluntários saudáveis.

Palavras-chave: Estimulação magnética transcraniana, Excitabilidade corticoespinhal, Neuroplasticidade, ΔPEM, Perturbação depressiva major

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Chapter 4

List of acronyms and abbreviations

ΔMEP	MEP mean amplitude change after rTMS
BDI-II	Beck Depression Inventory – II
CAML	Centro Académico de Medicina de Lisboa
CBT	Cognitive-Behavioural Therapy
CCU	Champalimaud Center for the Unknown
CSP	Cortical Silent Period
cTBS	continuous Theta-Burst Stimulation
DLPFC	Dorsolateral Prefrontal Cortex
EMG	Electromyography
FDA	Food and Drug Administration (USA)
FDI	First Dorsal Interosseous
fMRI	functional Magnetic Resonance Imaging
HAM-D	Hamilton Depression Rating Scale
ICF	Intracortical Facilitation
iTBS	intermittent Theta-Burst Stimulation
LICI	Long-Interval Cortical Inhibition
LTD	Long-Term Depression
LTP	Long-Term Potentiation
M1	Primary Motor Cortex
MAO-I	Monoamine Oxidase Inhibitors
MDD	Major Depressive Disorder
MEP	Motor Evoked Potential
MINI	Mini International Neuropsychiatric Interview
MT	Motor Threshold
OCD	Obsessive-Compulsive Disorder
PAS	Paired Associative Stimulation
QPS	Quadripulse Transcranial Magnetic stimulation
rTMS	repetitive Transcranial Magnetic Stimulation
SICI	Short-Interval Cortical Inhibition

List of acronyms and abbreviations (continuation)

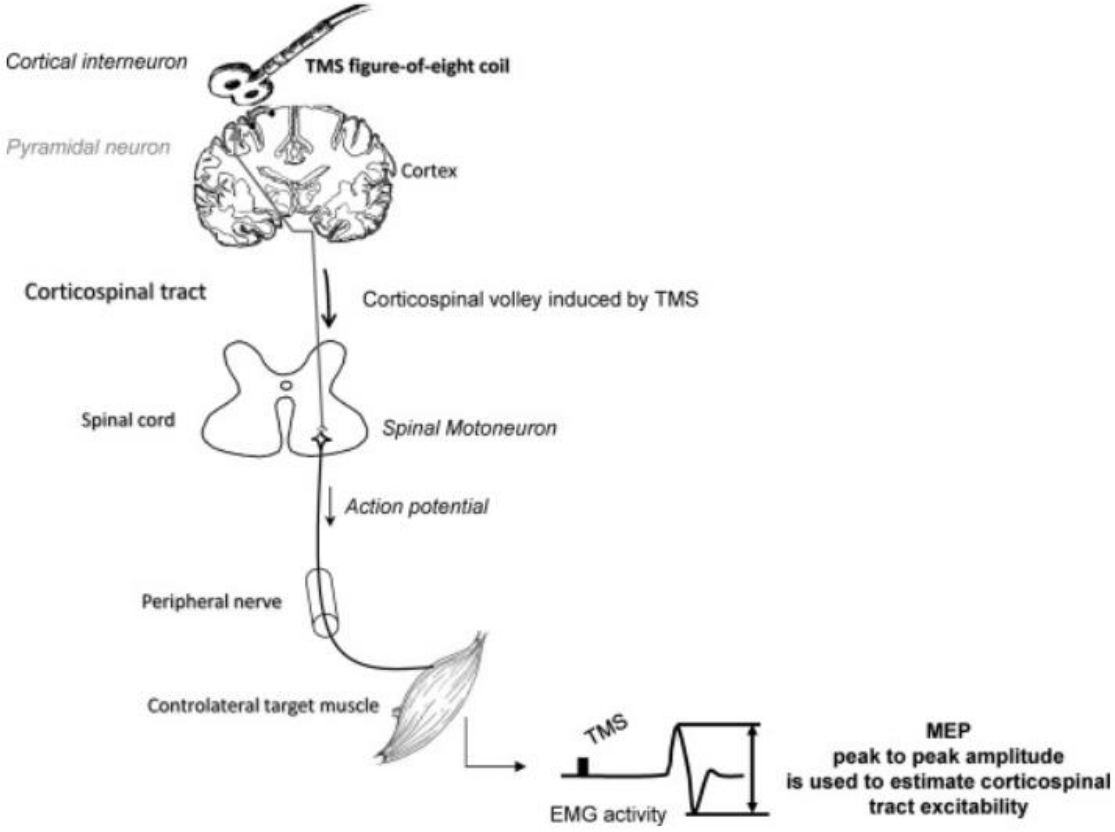
SSRI	Selective Serotonin Reuptake Inhibitors
TCA	Tricyclic Antidepressants
TMS	Transcranial Magnetic Stimulation
TRD	Treatment-Resistant Depression
WHO	World Health Organization

1. Introduction

1.1. Transcranial Magnetic Stimulation

Since the first report of direct non-invasive stimulation of the human motor cortex using a pulsed magnetic field (Barker, Jalinous & Freeston, 1985), extensive research has been developed using Transcranial Magnetic Stimulation (TMS), with the most diversified purposes. TMS is a non-invasive brain stimulation (NiBS) modality whose mechanism of action is based on Faradays' law of electromagnetic induction. By the transmission of a large and brief pulse of electrical current through loops of copper wire, magnetic fields that are perpendicular to the plane of the coil are generated, thus inducing an electric field in conductive tissue/material adjacent to the generated magnetic field. In the field of Neuroscience, TMS is used to generate electromagnetic fields that can penetrate scalp and skull, leading to neuronal activity once this field reaches cortical tissue (Figure 1.1) (Hallet, 2007).

Figure 1.1. Schematic representation of TMS mechanism of action as assessed by Electromyography (as represented in Klomjai *et al*, 2015)



In the early years of research using TMS, studies assessed mainly temporal aspects and amplitude of motor conductivity, through Motor Evoked Potentials (MEP), after stimulating the motor cortex. Nonetheless, with continuous development in technical aspects of TMS, rapidly other stimulation parameters were possible to apply, which led to a considerable expansion of research in this field. If, at first, only single-pulses were possible to administer, i.e., the generation of a single electric pulse and consequently, single magnetic fields, soon after, it was possible to deliver multiples pulses repeatedly. Repetitive TMS (rTMS) protocols lead to the finding of modulatory effects on the cortex's excitability for a period longer than the stimulation protocol (Pascual-Leone *et al*, 1999; Spronks, Arns & Fitzgerald, 2010). Nowadays, parameters such as frequency, intensity, number of pulses, stimulation protocol's length, number of sessions, interval between pulses, interval between trains of pulses and others, have variable impact in cortical excitability and, therefore, in our neurobiology, allowing for more versatility in tackling different research questions. The considerable number of possible parameters for excitability's assessment has allowed us to understand better, not only the healthy brain, but has also given us clues regarding the pathophysiology of certain disorders (e.g. Veronezzi *et al*, 2016; Benussi *et al*, 2017).

TMS has been applied in several different lines of research, as well as in diverse clinical settings. In fact, research using TMS has grown exponentially, with 67 TMS-related publications found in PubMed in 1990, 1488 in 2000 and 8699 in 2012 (Eldayef, Press & Pascual-Leone, 2013). Importantly, the FDA has cleared the use of rTMS for treatment-resistant depression (TRD) and more recently, obsessive-compulsive disorder (OCD). Furthermore, there is evidence that TMS may have therapeutic benefits in several other contexts, including schizophrenia (Kennedy, Lee & Frangou, 2018), migraine (Lan, Xiaoni, Xiangpen, Xiaoming & Peng, 2017) and neuropathic pain (Rossini *et al*, 2015).

i. Single and paired-pulse TMS

Single-pulse protocols are still the most widely used for the assessment of cortical excitability, especially in the primary motor cortex (M1), since it's the only cortical region where a direct output of the stimulation can be measured, using Electromyography (EMG). Through such protocols, we can measure Motor Threshold (MT) activation, i.e., the least intensity needed to obtain a physiological response to the stimulation of a certain

motor cortical region, responsible for the movement of a targeted muscle. MT is the baseline from which all stimulation-related parameters will be configured. In that sense, it is considered as 100% of the intensity that will be used for excitability measurements. (Eldayef *et al*, 2013; Rossini *et al*, 2015).

While MT is measured by a metric of administered intensity, Motor Evoked Potentials (MEP) are the physiological representation of the cortical stimulation, typically assessed on a hand muscle, through EMG. This physiological measure is represented by a biphasic wave generated in response to the TMS pulse, with measures such as latency and amplitude that can be extracted as markers of corticospinal excitability. Since these are measures with a certain amount of variability, it is recommended that analyses are based on the responses to multiple pulses, rather than only one (Rothwell, Hallett, Berardelli, Rossini & Paulus, 1999). In terms of the specific number of pulses needed to administer for a reliable MEP average, some authors have shown that the optimal number is around 21 for amplitude assessment (Chang *et al*, 2016). Calculations using MEP's can be performed through baseline-to-peak amplitude difference, peak-to-peak amplitude, area under the curve or latency of onset.

Besides single-pulse and repetitive protocols, TMS can also be applied as a pair of pulses separated by a given Interstimulus Interval (ISI) which modulates, instantaneously, motor cortical activity by augmenting or diminishing the intensity of the target muscle's contraction and its respective physiological signal. This variation is based on the ISI's length, where smaller ISI's (1-5ms) reduce the motor response to stimulation, called Short-Interval Cortical Inhibition (SICI) and the opposite when ISI's are longer (7-20ms), called Intracortical Facilitation (ICF). Paired-pulses excitability measures can be composed by even longer ISI's, which inhibits the motor cortical activity. These intervals tend to range from tens to hundreds of milliseconds and are called Long-Interval Cortical Inhibition (LICI) (Radhu, Blumberger & Daskalakis, 2016).

ii. rTMS

rTMS protocols are best known to modulate cortical activity beyond the stimulation period itself. Through variation in pulses' frequency, it is possible to modulate the cortex differentially, by inhibiting or facilitating neuronal excitability and consequent activity.

In this sense, frequencies equal or inferior to 1Hz tend to reduce MEPs' amplitude after a rTMS protocol. On the other hand, frequencies equal to or higher than 5 Hz have shown to increase the mean amplitude of MEP's, although this frequency cut-off is not entirely clear (Fitzgerald, Fountain & Daskalaskis, 2006). rTMS protocols have diverse applications, such as the manipulation of certain brain regions' activity for the study of behavioural parameters. This methodological strategy has been used as an attempt to imply certain brain region's activity as a having an important role for certain cognitive functions, suggesting a causal relationship between brain region functioning and behavioural output (Polanía, Nitsche & Ruff, 2018).

Therapeutic use of rTMS is also supported by scientific evidence for certain neuropsychiatric disorders, such as MDD, schizophrenia or OCD, among others. Normally, rTMS protocols are applied with different stimulation parameters. Diverse disorders require different stimulation parameters and different cortical target regions. In the case of MDD, the main target region is the Dorsolateral Prefrontal Cortex (DLPFC), in either the left or the right hemisphere. For each one, different stimulation parameters are indicated. In the left hemisphere, high-frequencies are applied, while in the right DLPFC low-frequencies are used, hypothetically in order to compensate for abnormal hypoactivity and excessive hyperexcitability, respectively, in MDD patients. Diverse Systematic Reviews and Metanalysis support the beneficial therapeutic effects from this type of NiBS, for facilitatory protocols targeting the left DLPFC, inhibitory protocols targeting the right DLPFC, or even bilateral stimulation (e.g. Muntz *et al*, 2019). Since its clearance by the FDA in 2008, the treatment parameters most commonly in use for the treatment of MDD are 3000 pulses at a frequency of 10 Hz, administered at an intensity of 120% of the MT to left DLPFC target (Kobayashi *et al*, 2017).

iii. Other applications using TMS

Many findings regarding neurotransmission assessed *in vivo* in humans were performed using single-pulse, paired pulse and repetitive TMS, typically in combination with pharmacological manipulations. Ranging from the NMDAR antagonist memantine, which leads to enhanced ICI and reduced ICF compared to placebo or amantadine, another NMDAR antagonist with additional effects on monoaminergic and cholinergic transmission as well as potassium channels, leading to significant dose-dependent

reduction of ICF and a significant increase of LICI, but not SICI, MT, MEP recruitment curves, cortical silent period (CSP) or peripheral excitability, when compared to placebo (Reis *et al*, 2006). Furthermore, Kaelin-Lang and collaborators (2002) found that increased MEP amplitudes in response to rTMS were blocked by the GABA(A) receptor agonist lorazepam, but not by the NMDAR antagonist dextromethorphan, for example. Reis *et al* (2002) reported that topiramate, which has broad activity as a sodium-channel blocker, GABA(A)-receptor agonist and a NMDAR antagonist, elicited a significant increase of LICI compared to placebo. TMS also has shown potential to generate biomarkers for disease, for instance, in the context of neurodegenerative diseases, where cortical excitability measures have shown different physiological patterns when comparing Alzheimer's patients, Frontotemporal Dementia's patients and healthy participants (Benussi *et al*, 2017). Furthermore, Veronezzi and collegues (2016) have shown different cortical excitability dynamics when comparing atypical and melancholic subtypes of Depression.

TMS is nowadays seen as a central tool to assess cortical excitability and its neurobiological and behavioural correlates, *in vivo* in human models. Most of the mentioned excitability measures have been associated with diverse neurobiological mechanisms, even though there is difficulty in establishing a reliable biological underpinning for TMS after-effects (George & Aston-Jones, 2010). Independently of science's scarce knowledge about TMS's mechanistic pathways, this technique has opened new avenues in treatment strategies and research paradigms, such as the development of biomarkers for neuropsychiatric disorders.

1.2. Modulation of cortical excitability as a *proxy* for plasticity-like phenomena

Although the effects of rTMS are evident in the treatment of certain neuropsychiatric disorders and its use as tool to assess Central and Peripherical Nervous System function has grown exponentially in the past three decades, the neurobiological mechanisms underlying the modulatory effects of rTMS are still poorly understood. One of the most frequently reported after-effects of TMS protocols is the induction of plasticity, namely through Long-Term Potentiation (LTP) and Long-Term Depression (LTD) (Cirillo *et al*, 2016). Plasticity is a fundamental phenomenon in the brain, since it is the process through which it is modified by the environment. Neuroplastic processes account for learning and

memory, and are fundamental to predict and obtain reward, to integrate sensory stimuli in previously formed perceptions about a certain aspect of the world or even to compensate for an insult. It happens at different levels, ranging from microstructures, up to networks (Feldman, 2009; Liu *et al*, 2013).

LTP and LTD are the best described mechanistic pathways through which synaptic plasticity happens. LTP arises from coincident neurotransmission activity, becoming robust as neuronal firing synchronizes. It has been associated mostly with glutamatergic transmission and is dependent on the activity of its receptors, such as NMDA and AMPA, resulting in increased synaptic efficacy. LTD represents the inverse process and results in a decrease of synaptic efficacy, through depotentiation of previously coincident synaptic activation, or *de novo* LTD, which is a depression of unpotentiated baseline synaptic activity. LTP and LTD can last weeks or even months after induction. These alterations encompass changes in molecular mechanisms, such as alterations in genes and proteins expression in the neuronal nucleus (Cooke & Bliss, 2006). Other processes fundamental for a homeostatic brain plasticity are neurogenesis, angiogenesis and gliogenesis, i.e., the generation of new brain cells, blood vessels and glial cells, respectively.

rTMS protocols seem to modulate brain plasticity, as reported in studies using animal models. Firstly, TMS initiates action potentials in neurons and/or changes the level of neural excitability and the cell membrane resting potential and threshold (Funke *et al*, 2011). Increased glutamatergic activity is thought to be a result of expression of NMDA receptors and long-lasting effects on arborization and morphology of dendritic spines at the apical dendrites of CA1 pyramidal neurons (Vlachos, Muller-Dahlhaus, Rosskopp, Lenz, Ziemann & Deller, 2012). Furthermore, there are reports of coordinated induction of Ca^{2+}-dependent changes of specific inhibitory post-synapses on principal neurons (Lenz *et al*, 2016). Once more, frequency seems to play a key role in modulating plasticity, whereas high-frequency protocols induce the previously mentioned changes, and low frequencies, the exact opposite effect, such as the increase in action potential thresholds, for instance (Cirillo *et al*, 2016).

However, assessing neuroplastic changes in humans, *in vivo*, is challenging. For that, TMS has properties that help researchers to study this paradigm noninvasively. Considering the assumptions present in the literature regarding TMS's potential for the

assessment of oscillations in cortical excitability as a correlate of neurotransmitters involved in neuroplasticity processes, studying its differences in healthy participants and comparing it with specific patient populations would be the natural logical step to imply cortical excitability measures as biomarkers for the disease. Nonetheless, results have been equivocal, where excitability measures have shown inconclusive patterns in disease when compared to healthy controls (Radhu *et al*, 2013; Bunse *et al*, 2014; Grunhaus *et al*, 2003). Furthermore, excitability measures have shown limited properties in predicting treatment response for TRD patients who underwent a DLPFC rTMS therapeutic cycle (Fitzgerald *et al*, 2004).

Nonetheless, NiBS protocols allow scientists to measure not only excitability itself, but also its modulation. For instance, by pairing TMS with EMG it is possible to establish a baseline of corticospinal excitability, through averaging peak-to peak amplitudes from MEPs. A modulatory rTMS protocol can then be applied, and a reassessment is made, measuring changes from before to after the modulation (e.g. Maeda *et al*, 2000). Such process is designated as cortical excitability modulation measurement and has been widely studied in healthy humans to understand how different stimulation parameters, such as frequency or number of pulses affect cortical excitability (e.g. Maeda *et al*, 2001). Modulatory measures have also been used to study differences in plasticity-like phenomena in health and disease (e.g. Kuhn *et al*, 2006). For example, given the proposal for neuroplasticity deficits as part of the pathophysiology of MDD, differences would be expected in cortical excitability modulation measures between MDD patients and healthy controls. This has been demonstrated using paired associative stimulation (PAS) – A modality of cortical excitability modulation - where the modulatory effect was inferior in MDD patients, when compared to healthy controls (e.g. Kuhn *et al*, 2016). Furthermore, this phenomenon was gradually heightened up to 60 minutes (Player *et al*, 2013). Similar results have been demonstrated using intermittent Theta-Burst Stimulation (iTBS) – a protocol for cortical excitability facilitation -, where up to 20 minutes, statistically significant corticospinal excitability modulatory differences are reported between drug-free MDD patients and healthy participants (Vignaud, Damasceno, Poulet & Brunelin, 2019).

Cortical excitability modulation measures have also been shown to have predictive value for treatment response, using facilitatory parameters, i.e., 10Hz rTMS delivered to M1.

The difference between pre- and post- rTMS MEP's mean amplitude was calculated (ΔMEP) and, afterwards, MDD participants were enrolled in an acute cycle of treatment using rTMS to the left DLFPC, with the same parameters as to the M1 rTMS protocol. Results showed that patients with higher ΔMEP values, before treatment initiation, responded better to rTMS treatment, as assessed through the variation in symptom severity scales before and after the treatment (Oliveira-Maia, Press & Pascual-Leone, 2017). Modulation of cortical excitability by rTMS thus seems to be a promising diagnostic and/or treatment biomarker, at least in the context of MDD.

1.3. Interhemispheric asymmetry in the context of major depressive disorder

MDD is a neuropsychiatric disorder with one of the highest levels of incidence and prevalence worldwide. It is a commonly occurring disease, can be recurrent and leads to reduced functional capacity, decreases in quality of life and medical comorbidities, such as diabetes mellitus, heart disease and stroke. Women are twice as likely to have the diagnosis when compared to men. Furthermore, in high-income countries, lower income citizens are more likely to suffer from the disease and for a longer period (Kessler & Bromet, 2013). The World Health Organization (WHO) estimates that, by 2020, MDD will be the second leading cause of disability worldwide, as assessed by the number of years lived with disability (Otte *et al*, 2016). Criteria for MDD diagnosis, according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) suggests that five or more cardinal symptoms must be present most days for at least 2 weeks, with a marked difference from previous levels of functioning. Some of the symptoms are depressed mood or diminished interest or pleasure, considerable decrease or increase in weight and/or appetite, insomnia or hypersomnia. psychomotor retardation or agitation, fatigue or loss of energy, feelings of worthlessness, diminished ability to think or concentrate and recurrent thoughts of death, suicide ideation or a specific plan to commit suicide. MDD can present itself with different specificities, such as anxious distress, melancholic features, psychotic features, peripartum onset or a seasonal pattern (American Psychiatry Association, 2013).

The most common forms of treatment for MDD are psychotherapy and pharmacotherapy, most commonly in the form of Cognitive-Behavioural Therapy (CBT) and Serotonin Selective Reuptake Inhibitors (SSRI), respectively. When combined, the two therapeutic

strategies account for response in approximately two thirds of patients, being more effective than when applied separately. Non-response among the remaining third of patients may result from inadequacy in therapeutic administration, poor diagnosis and/or insufficient knowledge about disorder mechanisms, among others (Pampallona, Bollini, Tibaldi, Kupelnick & Munizza, 2004; Driesen & Hollon, 2010; Rush *et al*, 2006). Many theories on the pathophysiology of MDD have been put to test, including the monoamine hypothesis, the inflammation hypothesis, chronic stress and impairment in neuroplasticity-related events (Otte *et al*, 2016). The latter phenomenon (i.e. neuroplasticity) has been shown in different study paradigms, ranging from animal models to *post-mortem* studies, in humans. For instance, cellular changes were observed in the hippocampus, with reduced pyramidal cell frequency and overexpression of inflammation markers in glial cells, when compared with healthy controls (Stockmeier *et al*, 2004). Furthermore, there is evidence for disturbances in glutamatergic and GABAergic receptors, such as NMDA, AMPA, GABAA and GABAB, impairing LTP and LTD (Murrough, Abdallah & Mathew, 2017), as well as evidence that ketamine, a NMDA-antagonist, ameliorates depressive symptoms, possibly through recovery of plasticity in dendritic spines of prefrontal circuitry (Moda-Sava, Murdock, Parekh, Fetcho, Huang, Huynh & Witztum, 2019). Nonetheless, this hypothesis remains challenging to tackle, specially using humans as a research model. TMS might be a powerful tool for these questions, since it is possible to assess excitability measures as well as their modulation, thought to be *proxy* measurements of glutamatergic and GABAergic activity, as well as of plasticity-like phenomena.

Besides impaired neuroplastic processes, in the context of MDD, the literature also suggests cortical activity asymmetries between hemispheres as a common phenotype in this clinical population. With a special incidence in frontal regions, using imaging techniques, such as functional Magnetic Resonance Imaging (fMRI) or EEG, a tendency for left hemisphere frontal hypoactivity and/or hyperactivity in the right hemisphere has been proposed as a potential neurobiological marker of MDD (Grimm *et al*, 2008; Herrington *et al*, 2010). NiBS methods have also been used in the study of interhemispheric asymmetries, particularly through the assessment of excitability measures from both M1 cortices. Patients with depression have a tendency for higher MT in left hemispheres when compared to right hemisphere, suggesting a higher electrical demand to elicit neuronal response in left hemisphere, when compared to the homologous

region (e.g. Concerto *et al*, 2013). Paired-pulse measures, show lower excitability in left M1 when compared to the right hemispheres of MDD patients (e.g. Lefaucheur *et al*, 2008). After treatment with left DLPFC rTMS there is evidence for an increase in left M1excitability, as well as a reduction in interhemispheric asymmetry, associated with symptomatic improvement and sustained treatment effects (Cantone *et al*, 2017). Following a similar rationale, Bajwa and collaborators (2008) studied the modulatory effects of a M1 rTMS protocol at 1 Hz on the left hemisphere to understand how ipsi- and contralateral stimulation effects would manifest, comparing MDD patients with healthy subjects. Results showed an inhibitory effect on the left hemisphere for both populations, but only healthy subjects presented a contralateral effect, i.e., cortical excitability facilitation. The authors interpreted the results as an absence of homeostatic induction of cortical activity between hemispheres in MDD patients.

Nonetheless, studies attempting to assess interhemispheric modulatory effects in healthy participants have not been able to establish a consistent pattern of the interhemispheric effects. 5 Hz and PAS stimulation protocols increased MEP's mean amplitude in the non-stimulated M1 (Gorsler *et al*, 2003; Shin & Sohn, 2011). On the other hand, iTBS protocols inducing cortical activity facilitation on the stimulated M1, induced an inhibitory effect on the contralateral motor cortex (Di Lazzaro *et al*, 2008). In what concerns inhibitory protocols, such as 1Hz M1 protocols or continuous Theta-burst (cTBS), both increments and decrements are reported throughout the literature, whether in the stimulated region as well as in the contralateral cortex (Tsutsumi *et al*, 2014). It could be hypothesised that different stimulation parameters induced different modulatory effects. In fact, Di Lazzaro and colleagues (2011) have tried to prove this idea by systematically testing 6 different modulatory protocols, especially in terms of distal effects. Only a Quadripulse Transcranial Magnetic stimulation (QPS) protocol induced a significant change in the contralateral M1, facilitating its excitability. Some results might be related to technical issues, since modulatory contralateral effects have been shown in cats using metabolic activity tracing, where the effect was as large as the amount of fibers connecting the stimulated region with the contralateral cortex (Valero-Cabré *et al*, 2005).

1.4. Influencing factors in measures of excitability and excitability modulation

Literature regarding excitability measures and their modulation report influence from diverse factors. These range from intrinsic individual features, such as age, gender or brain-to-skull distance, to procedure related factors just as coil placement and data acquisition parameters (e.g. sampling-rate frequency). Such variables need to be considered for the optimization of results. Throughout literature using rTMS, age seems to present itself as one of the most robust factors influencing excitability measures and its modulation (Bhandari *et al*, 2016). Age plays an important role in plasticity processes as well as in muscle strength and motor performance (Bashir *et al*, 2014). Certain reports highlight different cortical excitability patterns based on discretized groups at the age of 50, where participants 50 or more years old have smaller MEP's mean amplitude as well as smaller Cortical Inhibition when compared with a younger group (Cueva *et al*, 2016). Furthermore, impairment in modulatory induction through 1Hz M1 rTMS protocols has been shown in older participants, when compared to a younger cohort (Bashir *et al*, 2014). As an additional factor, some evidence suggests age as factor conditioning for rTMS treatment-response in depression (Kozel *et al*, 2000) as well as for the modulatory effects of rTMS in excitability (Grunhaus *et al*, 2003).

Gender has been thought as other factor influencing excitability. More precisely, it has been implied that, in women, sex hormones tend to affect excitability measures and to diverge according to the menstrual cycle. Excitability appears significantly increased from the early follicular phase to the late follicular phase and then decreased again in the luteal phase (Smith *et al*, 2002). Even though there seems to be evidence suggesting progressive changes in neurodevelopment gender-based differences, it is far from conclusive (Sun *et al*, 2015). In that sense, it is difficult to establish a clear-cut association between gender and excitability changes. Contrarily to age-related changes and to the best of my knowledge, gender has not been systematically studied to understand its potential effect and only sex hormones changes throughout women's menstrual cycle seem to be a prominent factor.

Equally to age and gender, circadian rhythms seem to play a role in excitability. Lang and colleagues (2011) studied diverse excitability measures and concluded an implication of time of the day in long-interval intracortical inhibition. The authors interpret these results

as a suppression of GABAergic neurotransmission throughout the day, but more direct evidence is needed in order to sustain that claim. Studies using EEG report a gradual sensitivity to TMS as the day progresses, present even after one night of total sleep deprivation. A considerable decrease is seen once the participants rest (Huber *et al*, 2012). Furthermore, more subtle findings are reported in what concerns circadian rhythms effects in TMS sensitivity, particularly stating robust circadian dynamics of cortical excitability in individual with highest endocrine markers of circadian amplitude. The authors conclude stating the importance for cortical excitability is the balance between circadian rhythmicity and sleep need, instead of sleep homeostasis alone (Ly *et al*, 2016).

Medication also seems to modulate excitability, as discussed above (see section on "Other applications using TMS"), and in greater detail in Minzenberg & Leuchter (2019). This also holds true for substances of common consumption, such as caffeine and nicotine. The available literature regarding such effects suggests enhanced corticospinal excitability in smokers that were acutely abstinent for the study's purposes, when compared to non-smokers (Grundey *et al*, 2013). On the other hand, evidence suggests impaired modulatory effects using PAS in chronic smokers, when compared to non-smokers, where MEP's means amplitude change after modulation is inferior in the experimental group when compared to controls (Lavender *et al*, 2019). For caffeine, results are inconsistent as some studies present no alteration in cortical excitability measures based on caffeine concentration changes (Orth *et al*, 2005), whilst others report an increase in cortical excitability after the intake of a caffeine dose comparable to one cup of coffee (Cerqueira *et al*, 2006). Even though there are many other factors that are hard to control, such as skull-to-brain distance or even the motor *homunculus* organization, those mentioned here can be assessed at the moment of physiological data acquisition.

2. Objectives

The present master thesis project is part of a broader project with the objective of understanding differences in ΔMEP mean amplitude when comparing healthy controls and MDD patients, in order to study this measure as a potential marker of neuroplasticity deficits in MDD. Thus, here I intended to optimize the protocol to assess this measure, and thus contribute towards standardization of such protocol for research and potential clinical purposes. My objectives were thus to confirm facilitation of corticospinal activity after 10Hz rTMS of the left M1, as demonstrated by Oliveira-Maia *et al* (2017) and Maeda *et al* (2000), while testing potential inhibitory effects of the contralateral M1, that have not been demonstrated conclusively in previous research. For protocol optimization, I also intended to understand how sociodemographic, clinical and protocol related variables affect the modulatory effects of motor cortex rTMS.

2.1. Hypotheses

My principal hypothesis is that motor cortical excitability will be facilitated after 10Hz rTMS of the left primary Motor Cortex. My secondary hypothesis is that activity in the contralateral primary Motor Cortex will be suppressed after 10Hz rTMS of the left primary Motor Cortex.

2.2. Specific aims

My work will be developed according to the following specific aims:

#1 Test modulation of mean MEP amplitude in the right First Dorsal Interosseous muscle after 10Hz rTMS of the left primary Motor Cortex (M1).

#2 Test modulation of mean MEP amplitude in the left First Dorsal Interosseous muscle after 10Hz rTMS of the left primary Motor Cortex.

#3 Explore how sociodemographic, clinical and procedure-related variables affect the ipsi- and contralateral effects of a 10Hz M1 rTMS protocol.

3. Methods

This study was approved by the ethics committees at the Champalimaud Centre for the Unknown (CCU) and Centro Académico de Medicina de Lisboa (CAML), in accordance with the declaration of Helsinky. Participants provided written informed consent where they were briefed in terms of the study purpose, possible side effects, and benefit in their participation. Nonetheless, briefing did not provide detail regarding the objectives of the study. Study objectives were only explained at the end of the session when participants were reimbursed for their travelling costs to the CCU.

3.1. Participants

Participants were healthy volunteers recruited consecutively at the CCU through informal announcement or through announcements for other ongoing studies at the Champalimaud Neuropsychiatry Unit.

i. Inclusion criterion

a. Participants needed to be healthy adults with ages ranging from 18 to 65 years old.

ii. Exclusion criteria

a. Eligibility for TMS was carefully assessed through a safety questionnaire adapted from Rossi *et al* (2009) and composed by items to inquiring about factors influencing the probability of side effects from TMS. This document included questions regarding history of epilepsy, loss of consciousness, hearing problems, metallic or magnetic implants, medication that might reduce the convulsive threshold as well as pregnancy status. If any of those parameters were present and were considered as endangering to the participant's health, they would not be enrolled.

b. Participants could not have any diagnosis of a mental disorder, such as MDD, history of bipolar or psychotic disorders, alcohol or other substance dependence or abuse, and moderate to severe suicide risk. MDD diagnosis was established

through Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I). The remaining domains were assessed through Mini International Neuropsychiatric Inventory (MINI) (Sheehan *et al*, 1998). Prior history of MDD was not an exclusion factor.

c. Participants could not have Central Nervous System disorders, such as Dementia, Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis or any structural and/or functional brain damage caused by a specific insult, such as Cerebrovascular Accident, Brain Tumour, a traumatic brain injury or Thrombosis. Participant could not also present Neurodevelopmental Disorders. All these elements were assessed by self-report.

d. Participants could not have any unstable medical disorder, which was assessed by self-report.

3.2. Procedure

Participants were asked to perform a session divided in 2 distinct moments, in the same day. Potential participants were approached, in a first instance, via phone or in person, where a short briefing of the study was presented and an informal assessment of eligibility conducted. If the potential participant was interested in participating and did not appear to meet exclusion criteria, a session was scheduled and an envelope was sent home with self-report materials, such as BDI-II, HCL-32, BIS-II, STAI-Y, OCI-R and WHO-5 (see below) and instructions to complete the questionnaires only one or two days before the session. When participants arrived at the CCU, the researcher would meet him/her, and accompany the participant to a first room where informed consent was signed, and clinical assessment was performed using SCID-I, MINI, HAM-D-17 and MoCA. After a short break, the participant was accompanied to a second room where the TMS protocol was performed.

3.3. Psychometric assessment

Psychometric assessment was performed to assess certain exclusion criteria as well as to understand how certain psychological constructs might associate with excitability parameters:

a. Mini International Neuropsychiatric Inventory (MINI) was used to assess mental illness diagnoses (Sheehan *et al*, 1998). MINI is a semi-structured clinical interview script, based on the criteria proposed by the Diagnostic and Statistical Manual of Mental Disorders (DSM) (American Psychiatric Association, 2013). The 5.0.0 version was used in this study, which is validated for Portuguese population and refers to the DSM-IV framework (Guterres, Amorim & Levy, 1999). Through this guided interview, it is possible to establish the diagnosis for disorders such as Major Depressive Disorder (present, past, recurrent and with melancholic features), Dysthymia, Suicide risk, (Hypo-)manic episodes, Panic Disorder, Agoraphobia, Social Phobia, Obsessive-Compulsive Disorder, Post-traumatic Stress Disorder, Alcohol and Substance dependence and abuse, Psychotic Disorders, Anorexia and Bulimia Nervosa, Generalized Anxiety Disorder and Anti-social Personality Disorder.

b. Structured Clinical Interview for DSM-IV Axis I (SCID-I) is semi-structured clinical interview script for the assessment of Axis-I Mental Disorders based in criteria proposed in DSM-V (First *et al*, 2004). For the purposes of this study, only MDD evaluation was performed using this guide and was used for the diagnosis of present and past episodes of MDD.

c. 17-item Hamilton Rating scale for Depression – HAM-D-17 is also a clinical semi-structured interview script, that intends to assess depressive symptoms' severity. It is considered the gold standard in mental health research and clinical practice for its purpose. Composed by 17 items that refer to symptoms such as depressed humour, guilt, suicidal intention, sleep habits, energy, motivation, psychomotor retardation, anxiety and somatic manifestations, hypochondriac thoughts, weight loss and insight towards the disease. Some items are scored based on participants/patient's explicit report while others also take into

consideration the interviewer's perspective. In this experiment a version validated for the Brazilian population (Moreno & Moreno, 1998) was adapted to European Portuguese. It generally takes 15-20 minutes to complete the interview and score the results. Eight items are scored on a 5-point scale, ranging from 0 = not present to 4 = severe. Nine are scored from 0-2 and the questions are asked considering the participant/patient's last week. Even though this was not an instrument considered for diagnosis, the cut-off to consider a depressive stated based on HAM-D-17 is 8-13 points on total score for mild depression, 14-18 for moderate depression, 19-22 for severe depression and 23 or more for very severe depression (Hamilton, 1960)

d. Beck Depression Inventory-II (BDI-II) is a self-report clinical tool to assess depressive symptom severity. The items assess sadness, pessimism, past failure, loss of pleasure, sense of guilt, sense of punishment, self-hatred, self-criticism, suicidal thoughts and ideation, cry, agitation, loss of interest, indecision, self-depreciation, loss of energy, sleeping habits, irritability, appetite, concentration difficulties, fatigue and loss of interest for sex. BDI-II is an instrument to assess self-rated depression severity. Scores of 0 to 13 indicates minimal depression, 14 to 19 indicates mild depression, 20 to 28 indicates moderate depression, and 29 to 63 indicates severe depression. It is composed by 21 items scored on a 4-point scale, ranging from 0 = not present to 3 = severe (Beck *et al*, 1996). The version used in this study was validated for the Portuguese population by Campos & Gonçalves (2011).

e. State-Trait Anxiety Inventory - Y (STAI-Y) (Spielberg *et al*, 1983) is an inventory based on a 4-point Likert scale and consists of 40 questions for self-report of anxiety. It is an instrument that allows the measurement of two types of anxiety: state, i.e., related to a certain timepoint of the person's life and trait, i.e., anxiety as a personal characteristic. In this study, a validated version for the Portuguese population was used (Santos & Silva, 1997).

f. Barratt Impulsiveness Scale – 11 (BIS-11) is composed by 30 items which assess impulsivity traits (Patton *et al,* 1995). Each item is scored in Likert scale ranging from 1 to 4, where 1 means "Never or Seldomly" and 4 "Almost always/Always".

In this study, a European Portuguese version was used (Cruz & Barbosa, 2012), based on a version validated for the Brazilian population (Malloy-Diniz *et al*, 2010).

g. Obsessive-Compulsive Inventory – Revised (OCI-R) is an inventory for self-assessment of obsessive and compulsive symptomatology (Foa *et al*, 2002). It is composed by 18-itens which, when clustered, evaluate six different factors present in patients with obsessive-compulsive symptoms: cleaning, verification, order, hoarding, obsessions and neutralization. Each item ranges from 0 to 4, being 0 "Nothing" and 4 "Extremely". OCI-R has been validated in non-clinical Portuguese samples (Cardoso & Faria, 2015) and was applied in this study to correlate obsessive-compulsive traits with excitability.

h. Hypomania Checklist – 32 (HCL-32) is a psychometric instrument for the screening of hypomanic episodes (Angst *et al* 2005). Even though it does not provide a formal diagnosis for bipolar disorder, it allows clinicians and researchers to apply a practical and useful screening tool for such symptoms. Its output is based on 32 dichotomous items that are summed for the total score in this inventory. For the present study, a validated version for the Portuguese population was used (Camacho & Almeida *et al*, 2018).

i. World Health Organization – 5 items (WHO-5) (World Health Organization, 1998; Topp *et al*, 2015) is a Short self-reported measure of current mental wellbeing. It is known to have adequate validity in screening for depression as well as to predict clinical outcomes. WHO-5 is composed by 5 items ranging from 0 to 5 each, where 0 represents "At no time" and 5 represents "All of the time". Total scores range from 0 to 25 and are multiplied 4. Results are interpreted on a scale from 0 to 100, where 0 represents worst imaginable well-being and 100 best imaginable well-being.

j. Montreal Cognitive Assessment (MoCA) was designed to detect mild cognitive impairment (Nasreddine *et al*, 2005). It is composed by 30 items evaluating multiple cognitive domains, such as working memory, visuospatial abilities and attention, for instance. Even though MoCA is an instrument applied in elder populations, in this studied it was used in order to train the protocol application

which intends to include clinical populations in the future, averaging higher ages. A version validated for the Portuguese population was used (Freitas *et al*, 2011).

3.4. TMS procedure

TMS stimulation was performed using the MagProX100 (MagVenture), with a figure of 8 coil. All TMS procedures were performed under the recommended guidelines proposed by the International Federation of Clinical Neurophysiology (Rossi *et al*, 2009; Rossini *et al*, 2015). Participants were seated comfortably in a chair with their right and left arms in a relaxed position on the arms of the chair. The first step was to clean the skin from the region overlapping the First Dorsal Interosseous (FDI), in both hands, using alcohol. Afterwards, Ag/AgCl cutaneous electrodes (24 mm) were placed over the target muscle in each hand as well as a reference in the left elbow. Few trials were run in different regions to assess signal variability, using different reference regions, such as the right elbow or the left and right radial styloid processes. No significant differences were observed as the basal EMG signal remained a flatline, independently of the chosen reference.

A lycra swimming cap was then placed on the participants' head and the medial sagittal line as well as the intertragus line were drawn on the cap to obtain the interception point. From there, 5cm were measured to each side on the intertragus line and anteriorly on the sagittal line, to define 3 additional points. Two diagonal lines were then drawn to connect each of the lateral points to the anterior point. Finally, starting in each of the lateral points, 2.5cm were measured in the antero-medial direction of each of the diagonal lines to mark, in each hemisphere, the point represents an initial estimate of the motor hotspot for that hemisphere. Finally, 4 points were marked in a radius of 0.5cm around that initial estimate, to provide additional testing points of the motor hotspot. TMS single-pulses were then applied on each side to define the motor hotspot in the Primary Motor Cortex (M1). The order of hemisphere assessment was randomized prior to the study beginning.

The TMS coil was then placed in contact with the participants scalp, with the handle pointing posteriorly at 45º relative to the middle sagittal line. Participants were instructed to be silent and to be relaxed but to make the effort of not falling asleep, in order to reduce possible excitability fluctuations due to these factors. These instructions were repeated at

several moments across the protocol. Acquisition of excitability measures started only after basal EMG signal was a flatline, i.e., once the participants were considered in a resting state. Motor hotspot was visually defined as the testing point leading to the strongest contraction in hand muscles contralateral to the hemisphere being tested, starting at 50% of the machine maximum output. If no response was obtained, stimulation was repeated after a 5% increment in intensity, until the minimum intensity needed to generate a muscle contraction was reached. Resting Motor Threshold (RMT) was then established for that motor hotspot as the minimum intensity needed to elicit a response of at least 50μV in the contralateral FDI, in 5 out of 10 TMS single-pulses, separated approximately by a 5 second interstimulus interval. RMT measurements started at 50% of the machine's maximum electrical output. If no response was seen, the output would be increased by 5% until a response of least 50μV appeared in the EMG. From there on 2% reductions of the machine output would be tested until an intensity where less than 5 pulses generated a response of at least 50μV. Finally, 1% increments would be tested to confirm the most adequate intensity.

MEPs were assessed in both hemispheres at 120% of the RMT, before and after rTMS of the left motor hotspot, and in the side order defined by randomization. A total of 31 single pulses were administered for each assessment of MEP in each hemisphere, using a variable ISI, selected randomly from a set of intervals ranging from 6 to 10 seconds, with an average of 8 seconds and standard deviation of 2 seconds. This number of pulses was selected to optimize robustness of the measure in accordance with previous research (Biabani *et al*, 2018). An interval of 30 seconds between acquisition for each hemisphere, in order to adjust the intensity to the respective hemisphere and to place the coil in the designated motor hotspot of the second hemisphere. MEP's. Finally, M1 rTMS was applied at the left motor hotspot according to the parameters used by Oliveira-Maia *et al* (2017): twenty 8-s long 10Hz stimulation trains at 90% RMT intensity, with 52 seconds inter-train intervals, resulting in 1600 pulses per session over 19 minutes. Immediately after the rTMS protocol, MEP amplitude was re-assessed, as performed prior to rTMS.

i. Data acquisition and extraction

Muscle activity was recorded using an in-house built EMG Arduino (Multisensor Acquisition Board – https://www.cf-hw.org/electronics/systems/acquisition-system; Champalimaud Scientific Hardware Platform), incorporating 6 monolithic dedicated ADCs (not multiplexed), with acquisition happening in the same nanosecond range for all the 6 analogue inputs, as well as 5 digital inputs and a reference channel. Data streaming to an offline computer was done through Bluetooth and acquired using BONSAI software (Lopes *et al*, 2015). Out of the 6 available channels, 2 were used to acquire physiological data from each hemisphere. Another analog channel was used to acquire direct input from the TMS machine signalling a pulse delivery. Physiological signal was amplified 400 times and sampled at 1000Hz rate, bandpass filtered in a range of frequencies from 0.1 to 500Hz.

BONSAI generated 4 files as the output of each session. Two binary (.bin) files contained the physiology of both moments of MEP assessment: one file for pre-rTMS MEPs and other for the post-rTMS MEPs. For each of these files, a corresponding excel file (.csv) with the timestamp of the physiological data was also generated. A Python (Python Software Foundation, 3.6) script was specifically prepared for the purpose of data conversion from the binary to the physiology files and peak detection for each MEP. Minimum and maximum values of each MEP were thus extracted to an excel file. In order to confirm for potentially erroneous performance of the peak detection algorithm, each MEP for each participant was visually supervised and, if peaks were not correctly performed, its value was corrected or, if needed, the MEP would be excluded from the analyses. Peak-to-peak differences were also corrected if any peak was incorrectly detected by the algorithm. Finally, for each block of MEP assessment, an average of measurements from eligible pulses was calculated.

3.5. Statistical methods

Statistical analyses were performed using SPSS version 25.0 (IBM corporation). Physiological data included variables repeatedly for each hemisphere hemispheres: MT, mean pre-rTMS MEP amplitude, mean post-rTMS MEP amplitude and ΔMEP amplitude. ΔMEP amplitude was calculated as the percentage change of mean MEP amplitude, using

the MEP mean amplitude from both moments, with positive values (MEP amplitude increase) reflecting facilitation of cortical excitability by rTMS, and negative values (MEP amplitude decrease) representing suppression:

$$\Delta\text{MEP} = \frac{(MEPamplitude_{post-rTMS} - MEPamplitude_{pre-rTMS})}{MEPamplitude_{pre-rTMS}} \, x \, 100$$

Data for continuous measurements is presented as the mean ± standard error of the mean (SEM). One-sample *t*-tests vs. 0 were performed to demonstrate the existence of a modulatory effects relative to baseline. Unpaired two-sample t-tests were performed to compare ΔMEP values in the left and right hemisphere. These tests were also used for exploratory comparisons with data from previous studies. The Levene's test for equality of variances was used and, whenever significant, equal variances were not assumed for the *t-test*. Univariate linear regression predictive models were performed to assess the influence of potential moderators on outcome variables. Models were composed by variables that may affect modulatory effects in both assessed hemispheres. A multivariable model was constructed with variables that were significant in univariate analyses. Age and gender were included in all models as *a-priori* defined moderators of interest. Pearson correlations were computed to examine the relationship between psychometric instruments and physiological data (α = 0.05). An α = 0.05 was set as a threshold of statistical significance for all analyses.

4. Results

4.1. Sociodemographic, clinical, physiological and procedure related variables

32 participants were enrolled in this study (65.6% Female, 33 ± 14 years old). Data regarding sociodemographic, clinical, physiological and procedure related variables can be found in Table 4.1. One participant is not included in all analyses, since physiological data was corrupted for the left hemisphere. Data is thus available for 31 participants in left hemisphere and 32 in the right hemisphere. Seven participants reported previous MDD episodes, as assessed through SCID-II. All were studied, according to the research plan, in order to explore differences in excitability relative to participants who were never depressed.

Table 4.1. Descriptive statistics for sociodemographic, clinical and protocol factors

Variables		**N**	**%**	**M**	**SD**	**Min**	**Max**
Age (Years)		32		33	14	21	65
Gender	Male	11	34.4				
	Female	21	65.6				
Handedness	Left	2	6.3				
	Right	30	93.8				
Cigarette consumption	Non-Smoker	23	71.9				
	Smoker	9	28.1	6	5.6	1	15
Coffee consumption	No	8	25.0				
	Yes	24	75.0	2.0	0.9	1	4
Taking any medication	No	20	62.5				
	Yes	12	37.5				
Family history of Neuropsych. dis.	No	19	59.4				
	Yes	13	40.6				
Previous Major Depressive Episode	No	25	78.1				
	Yes	7	21.9				
Interval between self-report assessment and TMS session (days)		32		0.8	0.7	0	2
HAM-D		32		1.7	1.6	1	9
BDI - II		32		3.31	4.1	1	22
WHO-5		32		71	12.2	48	92
STAI		32		62.0	13.6	42	101
STAI-T		32		29.8	8.4	20	48
STAI-S		32		32.0	8.9	21	56

(continues)

Table 4.1. Descriptive statistics for sociodemographic, clinical and protocol factors (continuation)

Variables		N	%	M	SD	Min	Max
BIS		32		52.9	7.3	40	68
OCI-R		32		13.2	10.0	1	35
HCL-32		32		11.3	6.8	1	25
MoCA		32		26.9	1.9	22	30
First assessed hemisphere	Left	19	59.4				
	Right	13	40.6				
Left Hemisphere **Motor Treshold** (Max. intensity)			46.4	7.5	33	60	
Right Hemisphere **Motor Treshold** (Max. intensity)			47.3	7.7	33	65	
Left hemisphere **MEP mean amplitude pre-rTMS** (μV)			1018.6	781.7	214.4	3953.4	
Right hemisphere **MEP mean amplitude pre-rTMS** (μV)			913.7	579.9	224.3	2174.7	
Left hemisphere **MEP mean amplitude post-rTMS** (μV)			1177.7	751.5	256.2	3017.6	
Right hemisphere **MEP mean amplitude post-rTMS** (μV)			749.8	394.9	185.7	1759.7	
Left hemisphere Δ**MEP** (%)			28.4	70.3	-46.2	306.0	
Right hemisphere Δ**MEP** (%)			-5,1	39.4	-55.8	94,8	

4.2. Correlations

ii. Correlations between psychometric scores

A considerable number of significant associations can be seen in the data between psychometric materials applied in this study's research protocols (Appendix 1). Instruments built to assess severity of depressive symptoms (i.e., BDI-II and HAM-D) correlated robustly (r = 0.809, $p < 0.001$), as expected. HAM-D score was also significantly associated with instruments to assess anxiety symptoms, namely the STAI-Y (r =0.5, $p = 0.004$), STAI-T (r = 0.4, $p = 0.048$) and STAI-S (r = 0.4, $p = 0.01$). BDI-II also presented similar correlations with STAI-Y (r = 0.7, $p < 0.001$), STAI-T (r = 0.6, $p < 0.001$) and STAI-S (r = 0.4, $p = 0.02$). Furthermore, BDI-II correlated significantly with OCI-R (r = 0.4, $p = 0.03$). Also as expected, STAI-Y correlated significantly with its subscales STAI-T (r = 0.8, $p <0.001$) and STAI-S (r = 0.8, $p < 0.001$), but these subscales

did not correlate significantly with each other (r = 0.232, p = 0.201). STAI-Y correlates significantly also with BIS-11 (r = 0.572, p < 0.001). Finally, STAI-T correlates significantly with OCI-R (r = 0.349, p = 0.05) and HCL-32 (r = 0.431, p = 0.014). MoCA established exclusively negative associations. Statistical significance was achieved with BDI (r = -0.4, p = 0.02), STAI-Y (r = -0.5, p = 0.008), STAI-T (r = -0.4, p = 0.02) and BIS-11 (r = -0.5, p = 0.009). BIS-11 correlated significantly with OCI-R (r = 0.5, p = 0.007) and OCI-R associates with HCL-32 (r =0.4, p = 0.049). WHO-5 tended to correlate negatively with almost all other psychometric instruments, but with statistical significance only for the association with OCI-R (r = -0.4, p = 0.01).

iii. Correlations between excitability measures

Correlation tests between our physiological data (Appendix 2) show a significant and strong correlation between Motor Thresholds from each hemisphere (r = 0.8, p < 0.001). Pre-rTMS mean MEP amplitude in each hemisphere was also significantly associated (r = 0.5, p = 0.006; Figure 4.1), as was pre- vs. post-rTMS corticospinal excitability in either hemispheres (left: r = 0.8, p < 0.001; right: r = 0.8, p < 0.001; Figures 4.2 & 4.3). Post-rTMS mean MEP amplitude in each hemisphere was also significantly correlated (r = 0.7, p < 0.001; Figure 4.4). Finally, a statistically significant association was found between pre-rTMS right side MEP mean amplitude and ΔMEP in the same hemisphere (r = -0.5, p = 0.002; Figure 4.5).

Figure 4.1. Correlation between pre-rTMS MEP amplitude in the left and right hemispheres

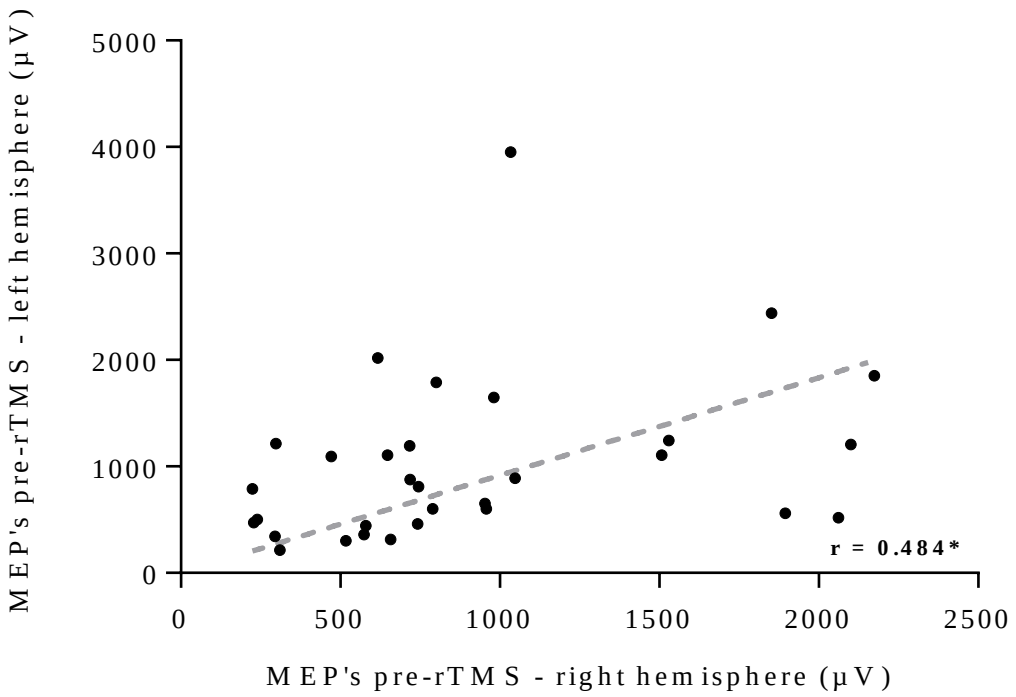


Figure 4.2. Correlation between left hemisphere pre- and post-rTMS MEP amplitude

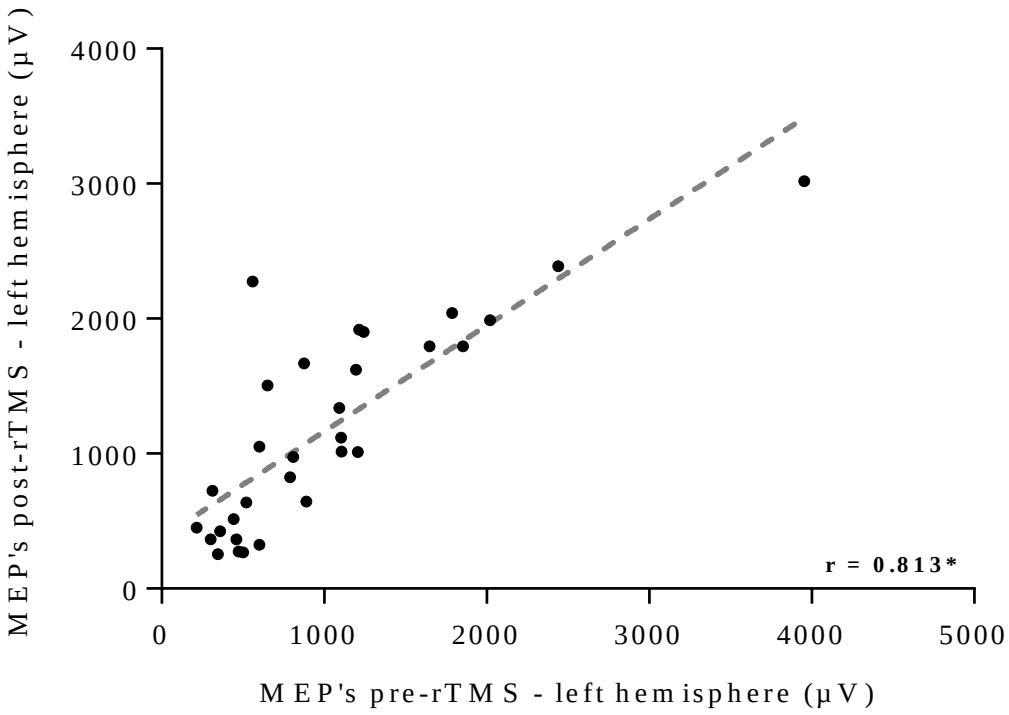


Figure 4.3. Correlation between right hemisphere pre- and post-rTMS MEP amplitude

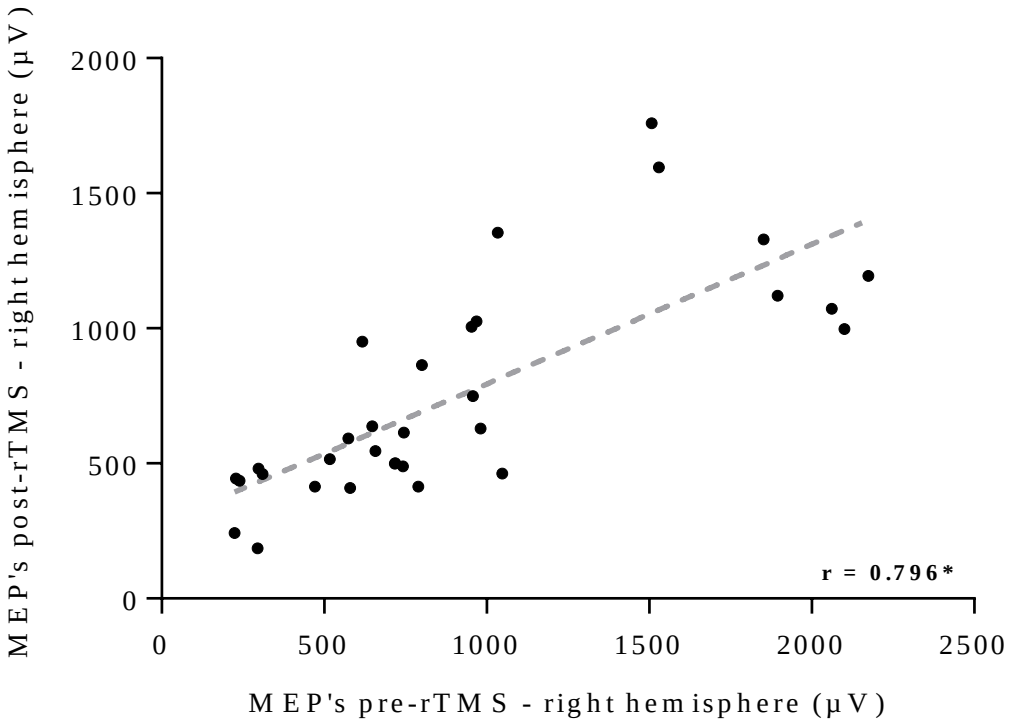


Figure 4.4. Correlation between post-rTMS MEP amplitude in the left and right hemispheres

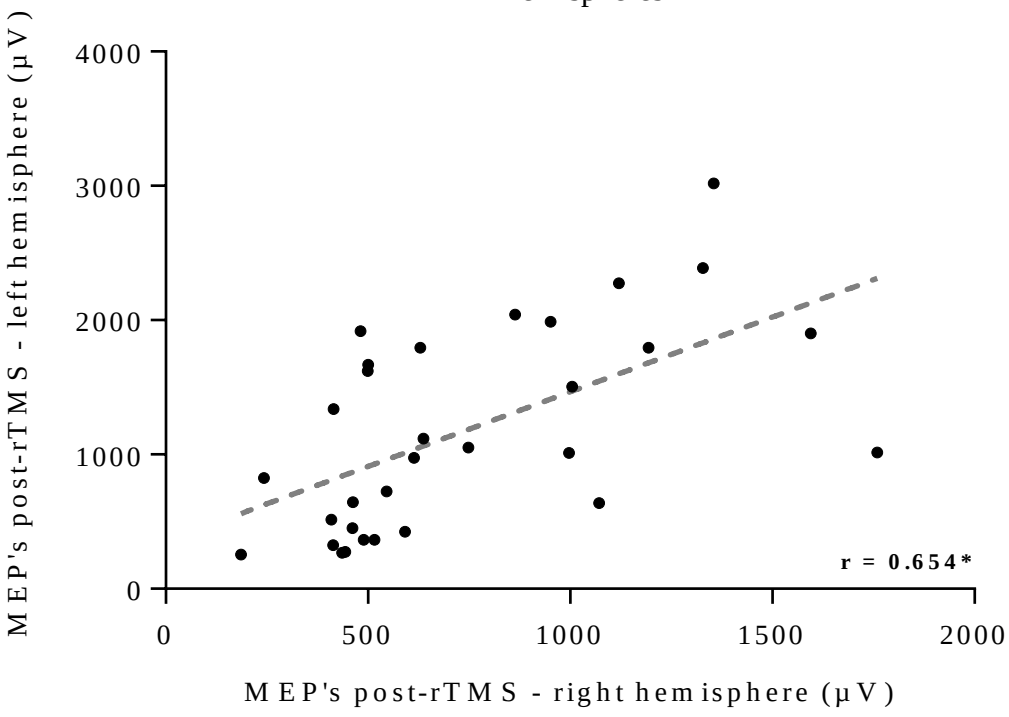
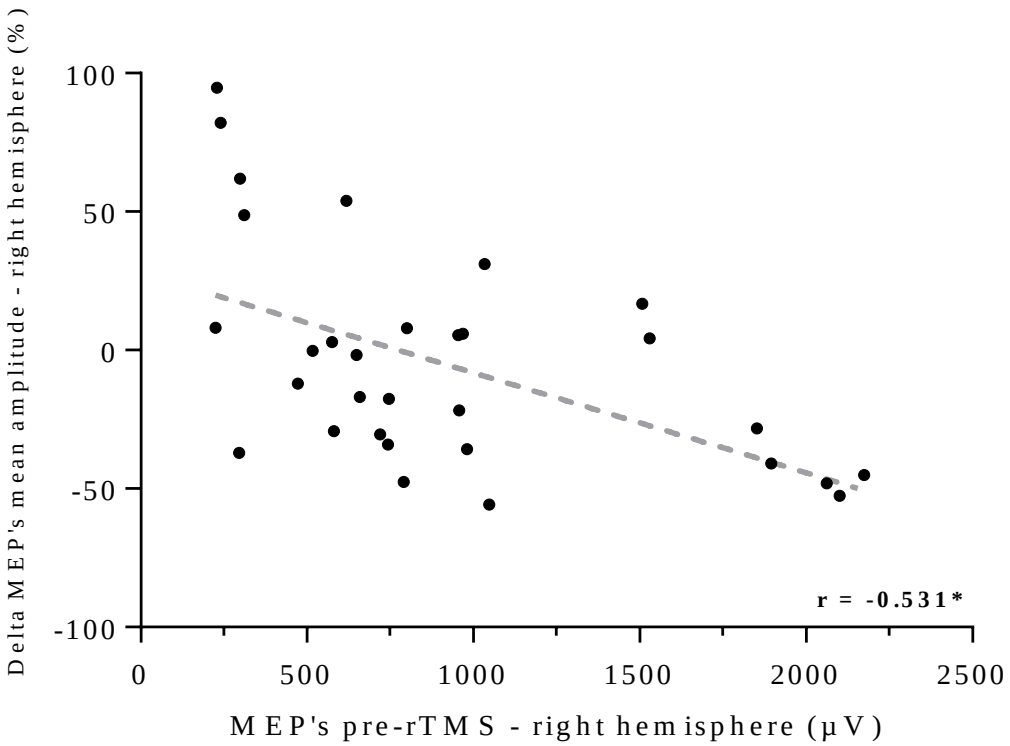


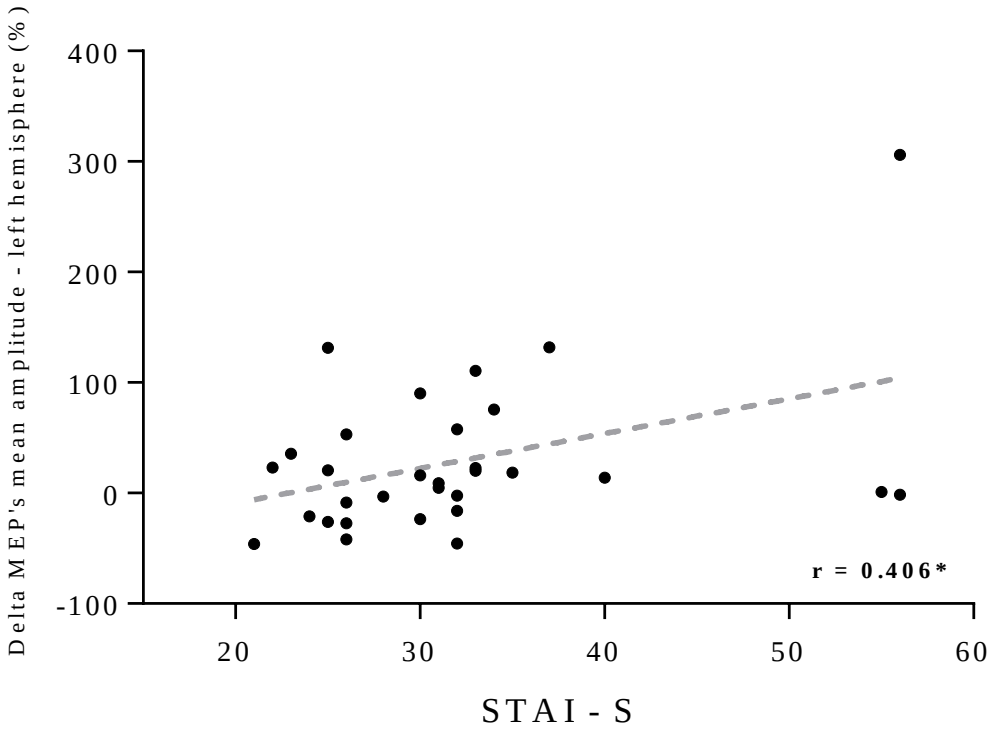
Figure 4.5. Correlation between right hemisphere pre-rTMS MEP amplitude and ΔMEP



iv. Correlations between psychometric scores and excitability measures

In tests of association between psychometric instruments and physiological measures (Appendix 3), STAI-S had a statistically significant association with left hemisphere ΔMEP (p = 0.4, r = 0.02) and a borderline association with post-rTMS mean MEP amplitude on the same side (r = 0.4, p = 0.05, Figure 4.6). MoCA had a statistically significant associations with pre-rTMS mean MEP amplitude on the left (p = -0.5, r = 0.01) and right hemispheres (p = -0.4, r = 0.03) and post-rTMS mean MEP amplitude on the left hemisphere (p = -0.5, r = 0.003).

Figure 4.6. Correlation between STAI state and ΔMEP on the left hemisphere

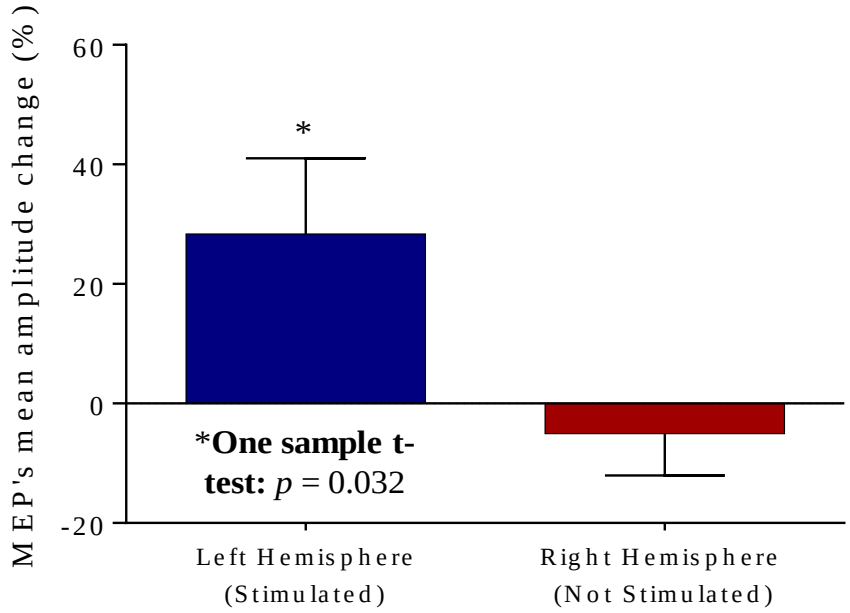


4.3. Cortical excitability modulation

i. ΔMEP on both hemispheres

To explore the effect of a 10Hz rTMS protocol on the left M1, a one-sample *t*-test vs. 0 was performed for ΔMEP on the left hemisphere. Significant modulatory effects on the stimulated M1 were found (t = 2.246, p = 0.032), confirming cortical excitability facilitation on that brain region (M = 28.4%, SEM = 12.6%). Conversely, regarding the contralateral M1, no statistically significant modulatory effect was seen (t = -0.726, p = 0.473), suggesting absent cortical excitability changes on the hemisphere contralateral to stimulation (M = -5.1%, SEM = 6.9%; Figure 4.7).

Figure 4.7. ΔMEP assessed on both M1 after a left M1 10 Hz rTMS protocol



ii. Predictive models for ΔMEP

Variables used to create multivariate predictive models for the modulatory effect in both hemispheres were chosen based on equivalent univariate linear regressions to predict ΔMEP values in each hemisphere (Tables 4.2 & 4.4). The variables used for the univariate models were descriptive of either demographics, health/clinical variables, and research protocol related factors. Age and gender were included in the multivariate models as *a priori* variables of interest. For the multivariate model built to predict ΔMEP of the left M1, in addition to age and gender, the number of cigarettes/day (β = 12.3, SE = 3.5, p = 0.001) and STAI-S score (β = 3.1, SE = 1.3, p = 0.02) were significant in univariate models and thus were included. The multivariate linear regression computed to predict left hemisphere's ΔMEP explains 30.1% of its variance (Table 4.3).

Table 4.2. Univariate linear regressions for left ΔMEP

Variable	β	SE	p	R^2
Age	1.386	0.868	0.121	
Gender	6.550	27.457	0.813	
Smoking status	**12.258**	**3.463**	**0.001***	**.278**
Coffee consumption	-6.312	11.128	0.575	
On medication	-38.973	25.365	0.135	
Family history of neuropsychiatric disorders	-2.484	26.033	0.925	
Previous MDD episode	-3.384	32.514	0.918	
Medical disease	-21.269	30.473	0.491	
HAM-D	0.673	7.930	0.933	
BDI	-1.463	3.128	0.643	
WHO-5	-.079	1.090	0.943	
STAI-T	0.195	1.531	0.899	
STAI-S	**3.143**	**1.315**	**0.024***	**.136**
STAI-Y	1.428	0.906	0.126	
BIS-11	1.609	1.746	0.364	
OCI-R	-0.542	1.289	0.677	
HCL-32	-0.203	1.908	0.916	
MoCA	-2.937	7.103	0.682	
Left pre-rTMS mean MEP amplitude	-0.022	0.016	0.186	
Left Motor Treshold	1.391	1.771	0.439	
Hemisphere assessed first	-31.822	25.357	0.220	
Time of assessment	-4.433	3.655	0.235	

Table 4.3. Multivariate predictive model for left ΔMEP

Variables	β	SE	p	Model	
				R^2	p
Age	0.5	0.8	0.5	0.301	0.009*
Gender	-5.6	24.6	0.8		
Cigarettes/day	11.8	3.5	0.002		
STAI-S	1.3	0.8	0.1		

For the contralateral (right) hemisphere, a separate predictive model was generated and, in addition to age and gender, right-side MEP mean amplitude before the rTMS protocol (β = -0.04, SE = 0.01**,** p = 0.002) and the order by which the hemispheres are assessed (β = 34.9, SE = 12.9**,** p = 0.01) reached statistical significance in predicting right-side ΔMEP

in univariate models, and were thus included in the multivariate model. The multivariate linear regression computed to predict right hemisphere ΔMEP explains 34.6% of its variance (Table 4.5).

Table 4.4. Univariate linear regressions for right ΔMEP

Variable	β	SE	p	R^2
Age	0.103	0.506	0.841	
Gender	-10.288	14.797	0.492	
Smoking status	-0.799	1.822	0.664	
Coffee consumption	-3.035	6.069	0.621	
On medication	-1.047	14.632	0.943	
Family history of neuropsychiatric disorders	16.668	14.100	0.246	
Previous MDD episode	22.340	16.644	0.190	
Medical disease	10.334	17.033	0.549	
HAM-D	-0.261	4.393	0.953	
BDI	-0.218	1.755	0.902	
WHO-5	-.112	0.590	0.851	
STAI-T	-.161	0.857	0.852	
STAI-S	.200	0.804	0.805	
STAI-Y	0.025	.529	0.962	
BIS-11	-0.290	0.976	0.769	
OCI-R	0.231	0.722	0.751	
HCL-32	-0.583	1.046	0.582	
MoCA	2.326	3.879	0.553	
Left pre-rTMS mean MEP amplitude	**-0.036**	**0.011**	**0.002***	**0.259**
Left Motor Treshold	0.380	0.931	0.686	
Hemisphere assessed first	**34.940**	**12.937**	**0.01***	**0.169**
Time of assessment	-0.777	2.064	0.709	

Table 4.5. Multivariate predictive model for right ΔMEP

Variables	β	SE	p	Model	
				R^2	p
Age	0.785	0.454	0.095	0.346	0.003*
Gender	-17.323	13.038	0.195		
Pre-rTMS MEP mean amplitude	-0.033	0.012	0.008		
Order of assessment	24.264	12.890	0.071		

iii. Factors influencing ΔMEP

As mentioned previously, in tests of linear association between psychometric and physiological measures, and consistently with regression analyses, only STAI-S had a statistically significant association with left hemisphere ΔMEP (p = 0.4, r = 0.02). To further explore variables influencing the modulatory effects of rTMS, namely demographic and clinical variables, group analyses were performed for a diverse number of factors, for both the stimulated and non-stimulated hemisphere, to understand with greater detail how each element might influence cortical excitability modulation. For that, dichotomous variables were chosen and unpaired two-sample *t*-tests performed. Test results are detailed below, for the left (Table 4.6) and right hemispheres (Table 4.7). The dichotomous factors chosen for analysis were: gender, smoking-status, coffee drinking-status, medication-status, prior history of MDD, family history of neuropsychiatric disorders, the side order for MEP's assessment and the moment of the day of the TMS session took place (morning/afternoon). Statistically significant differences were found only for ΔMEP on the right (non-stimulated) hemisphere, regarding the order for MEP assessment, i.e., participants with MEP assessment starting in the right hemisphere present higher and positive values for modulatory effects (M = 15.7, SEM = 12.6), whereas participants starting in the left hemisphere present values suggesting a negative modulatory effect (M = -19.3, SEM = 6.4; t = -2.5, p = 0.02).

Table 4.6. Group differences in ΔMEP (%) on the stimulated hemisphere (left)

Variables		**N**	**Mean**	**SEM**	***t***	***df***	***p***
Gender	Female	21	30.5	6.7	-0.2	29	0.8
	Male	10	23.9	18.6			
Smoking status	Yes	8	54.5	40.3	-0.8	7.9	0.4
	No	23	19.3	9.9			
Coffee	Yes	23	34.1	15.9	-0.8	29	0.5
Consumption	No	8	11.8	18.1			
On Medication	Yes	12	4.5	8.8	1.8	24.6	0.08
	No	19	43.5	19.2			
Family history of	Yes	13	26.9	16.1	0.1	29	0.9
Neuropsych. dis.	No	18	29.4	18.8			
Hemisphere	Left	18	41.7	19.4	1.3	29	0.2
assessed first	Right	13	9.9	12.8			
Time of	Morning	18	45.1	19.9	-1.8	24.3	0.08
assessment	Afternoon	13	5.2	9.9			
Previous Major	No	25	29	14.8	0.1	29	0.9
Depressive episode	Yes	6	25.6	23.9			

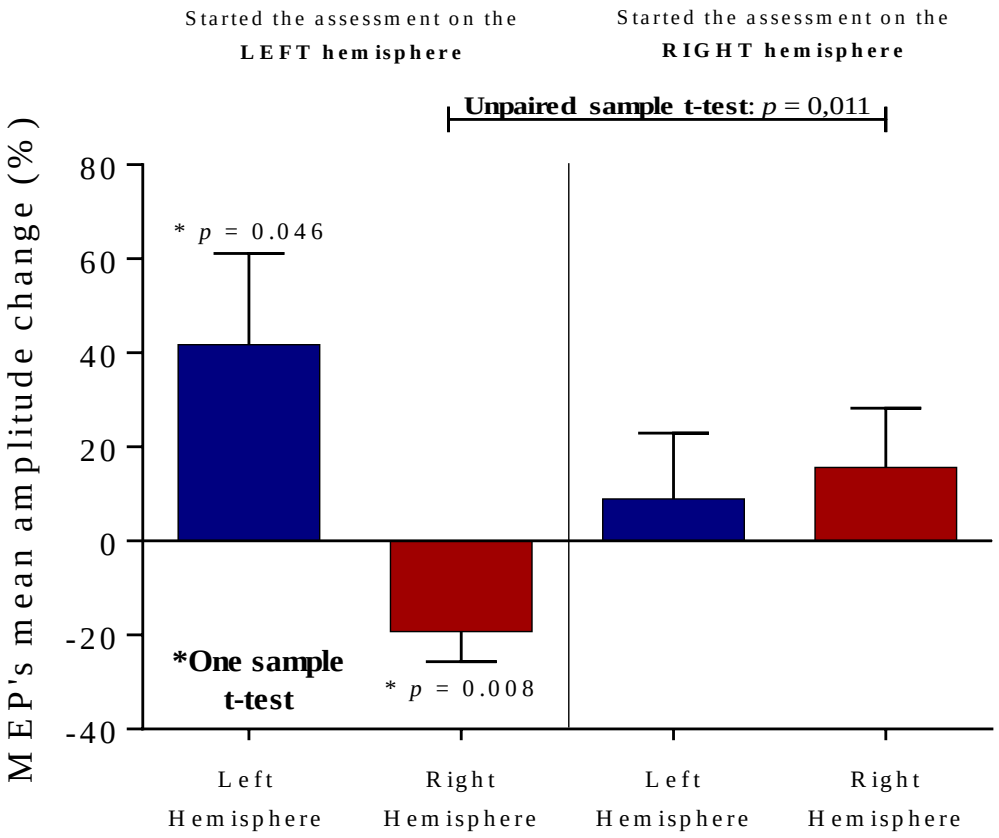
Table 4.7. Group differences in ΔMEP (%) on the non-stimulated hemisphere (right)

Variables		**N**	**Mean**	**SEM**	***t***	***df***	***p***
Gender	Female	21	-8.6	7.5	0.7	30	0.5
	Male	11	1.7	14.6			
Smoking status	Yes	9	-3.3	13.8	-0.2	30	0.9
	No	23	-5.8	8.7			
Coffee	Yes	24	-8.6	7.1	0.9	30	0.4
Consumption	No	8	5.5	18.5			
On Medication	Yes	12	-5.7	13.5	0.07	30	0.9
	No	20	-4.7	7.9			
Family history of	Yes	13	4.8	12.7	-1.2	30	0.2
Neuropsych. dis.	No	19	-11.83	7.82			
Hemisphere	Left	**19**	**-19.3**	**6.4**	**-2.5**	**18.3**	**0.02***
assessed first	Right	**13**	**15.7**	**12.6**			
Time of assessment	Morning	18	-4.5	8.3	-0.09	30	0.9
	Afternoon	14	-5,8	12.2			
Previous Major	No	25	-9.9	14.8	-1.3	30	0.2
Depressive episode	Yes	6	12.4	20.9			

One of the main aims of the present work was to optimize the research protocol in order to improve the quality of ΔMEP measurements in future studies. In that sense, the scope was deepened on the influence of variables which are controllable in ΔMEP values for both hemispheres. For that, two variables were selected, one of which already shown to influence ΔMEP, i.e., hemisphere order for MEP assessment. The second factor is the moment of the day at which the TMS session took place.

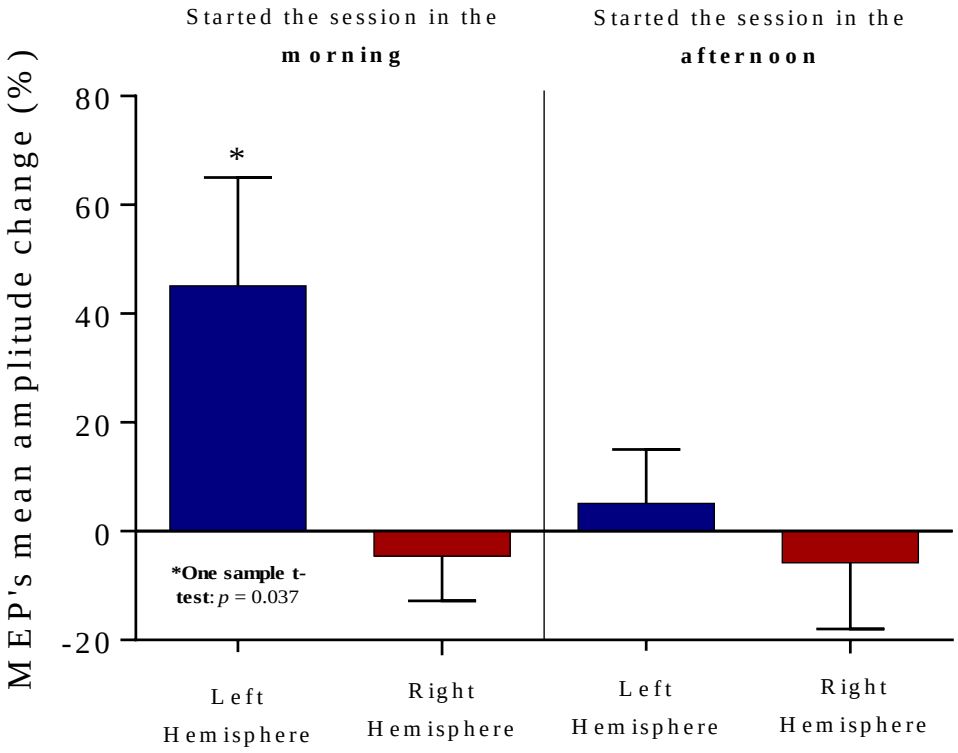
Considering the side order for MEP assessment one-sample t-tests were performed vs. 0 to assess significance of modulation (Figure 4.8). For left ΔMEP, in participants starting physiological assessment in the left hemisphere (M = 41.7%, SEM = 19.4%), a significant effect was found (t = 2.2, p = 0.046), suggesting a facilitatory effect. Conversely, in participants who started assessment on the right hemisphere (M = 9.9%, SEM = 12.8%), modulatory effects were not statistically significant (t = 0.8, p = 0.5). For ΔMEP values from the non-stimulated hemisphere, those starting assessment on the left hemisphere had a significant modulatory effect (M = -19.3%, SEM = 6.4%; t = -2.99; p = 0.008). while participants who started assessment on the right did not demonstrate a statistically significant effect (M = 15.7, SEM = 12.6%; t = 1.2; p = 0.2).

Figure 4.8. ΔMEP differences based on <u>first assessed hemisphere</u> (Mean ± SEM)



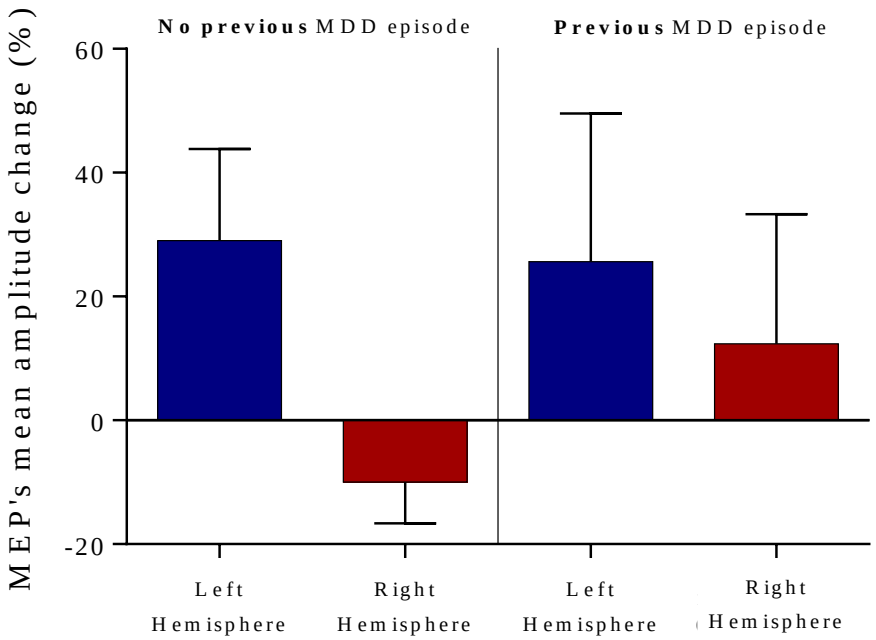
Our second variable of interest, i.e., the moment of the day at which the TMS session took place (Morning/Afternoon), through unpaired two-sample *t*-tests, show no differences between groups, both on the left and right hemisphere, as presented above. Nonetheless, by fractioning the sample based on this dichotomous variable and performing one-sample *t*-tests, statistically significant modulatory effects are only seen in the stimulated hemisphere on the group of participants who underwent the TMS session in the morning (M = 45.2%, SEM = 19.9; t = 2.3, $p = 0.04$; Figure 4.9).

Figure 4.9. ΔMEP differences based on session's moment of the day (Mean ± SEM)



There was also interested in understanding if a history of a previous depressive episode would impact the effects of rTMS. SCID was used to define history of MDD and in 32 participants, 7 were positive for prior MDD. In that sense, an opportunity to explore differences in cortical excitability modulation between subjects with and without previous depressive episodes emerged and an exploratory analysis was carried out. Unpaired two-sample *t*-tests were performed both for left and right ΔMEP (Figure 4.10) and statistically significant differences were not found, as described above (Tables 4.6 & 4.7).

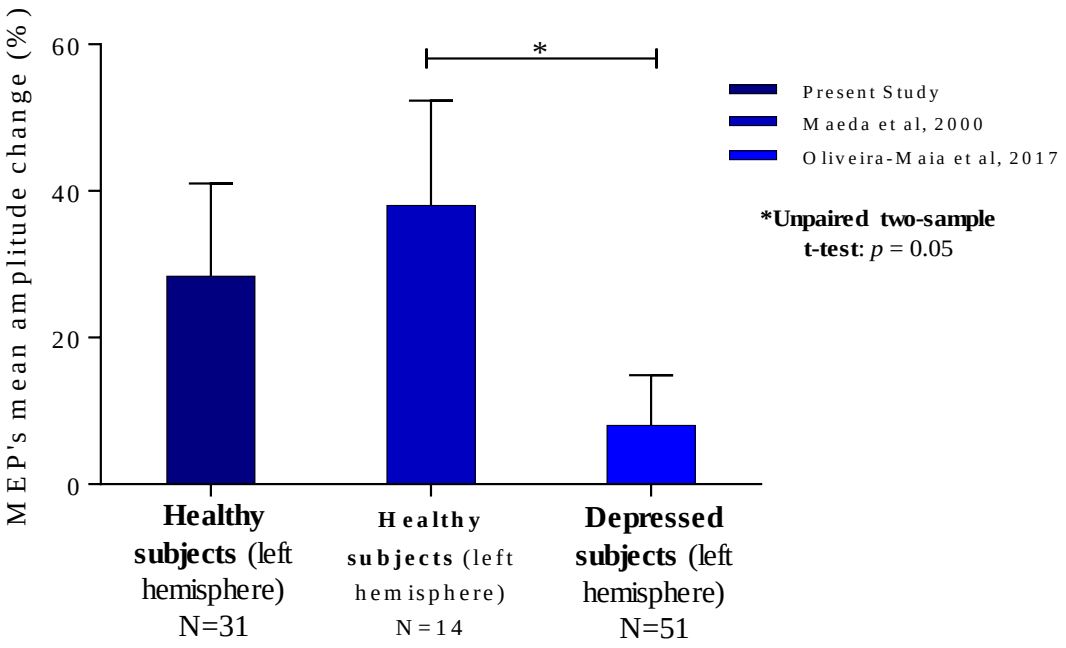
Figure 4.10. ΔMEP differences between participants with and without previous MDD episodes (Mean ± SEM)



iv. Exploratory comparisons with previous studies

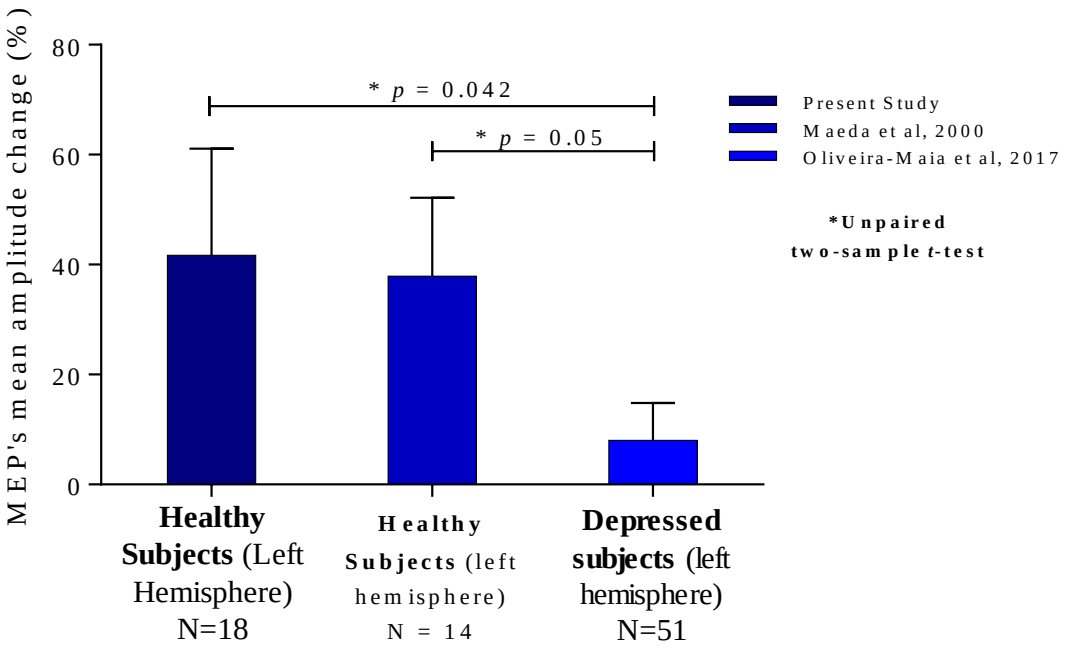
In order to assess similarities with results from other studies using identical TMS parameters, unpaired two-sample *t*-tests were performed, using the mean, standard deviation and sample sizes reported in Maeda *et al* (2000) and Oliveira-Maia *et al* (2017). The first study includes 14 healthy subjects and the latter 51 depressed patients, assessed before a DLPFC rTMS treatment cycle. Both studies modulated cortical excitability and assessed its effects only on the left M1, and thus such comparisons only regard this hemisphere. Oliveira-Maia *et al*, had already performed an exploratory comparison between their results and the Maeda *et al* results, showing a borderline statistically significant difference ($p = 0.05$) between depressed patients (M = 8%, SEM = 6.9%) and healthy subjects (M = 37.9%, SEM = 16.2%). When comparing results from the present study (M = 28.4%; SEM = 12.6%) with the 14 healthy subjects from Maeda *et al* (M = 37.9%, SEM = 16.2%), a statistically significant difference did not emerge (t = 0.4; $p = 0.6$), suggesting similar modulatory effects on the left M1 from both samples of healthy subjects. Nonetheless, when comparing results from the present study with the 51 depressed patients from Oliveira-Maia *et al* (M = 8%, SEM = 6.9%), statistically significant differences were also not found (t = 1.5, $p = 0.1$; Figure 4.11).

Figure 4.11. ΔMEP differences between present study, Maeda *et al* (2000) and Oliveira-Maia *et al* (2017) results (Mean ± SEM)



In a second indirect comparison, analyses were restricted to participants who started excitability assessment in the left hemisphere. Using only this subset of participants, unpaired two-sample *t*-tests show a significant difference (t = 2.1, $p = 0.04$) between this study's sample (M = 41.7%, SEM = 19.4%) and depressed patients enrolled in Oliveira-Maia *et al*, suggesting an increased facilitatory effect in these healthy participants, when compared to depressed patients. Again, comparisons of the this study's results with Maeda *et al*, did not reveal statistically significant differences (t = 0.1, $p = 0.9$; Figure 4.12). On the other hand, when analysing data from the subset of participants who started assessment on the right hemisphere (M = 9.9%, SEM = 12.8%), significant differences were not found in comparison to Oliveira-Maia *et al* (t = 0.1, $p = 0.9$) nor Maeda *et al* (t = 1.4, $p = 0.2$).

Figure 4.12. ΔMEP differences between participants whose assessment started on the left hemisphere, Maeda *et al* (2000) and Oliveira-Maia *et al* (2017) results (Mean ± SEM)



Analyses were then restricted to the subset of participants who did their TMS session in the morning. As can be seen in Figure 4.13, our subset (M = 45.2%, SEM = 19.9%), when in comparison with depressed patients from Oliveira-Maia *et al*, present a statistically significant increase of modulatory effects (t = 2.3, p = 0.03), but no differences in comparisons with healthy subjects recruited by Maeda *et al* (t = 0.3, p = 0.8). When considering the subset of participants who were tested in the afternoon (M = 5.1%, SEM = 9.9%) differences relative to Oliveira-Maia *et al* (t = 0.2, p = 0.8) and Maeda *et al* (t = 1.8, p = 0.09) were, not statistically significant.

Finally, comparisons restricted to previously depressed patients, as defined by SCID, in our sample (M = 25.6%, SEM = 23.9%) did not reveal differences relative to healthy volunteers in Maeda *et al* (t = 0.4, p = 0.7) nor depressed patients in Oliveira-Maia *et al* (t = 0.8, p = 0.4), even though this study's sample of previously depressed patients has a considerably higher modulatory value on the left hemisphere, when compared to currently depressed patients (Figure 4.14).

Figure 4.13. ΔMEP differences between participants whose session started in the morning, Maeda *et al* (2000) and Oliveira-Maia *et al* (2017) results (Mean ± SEM)

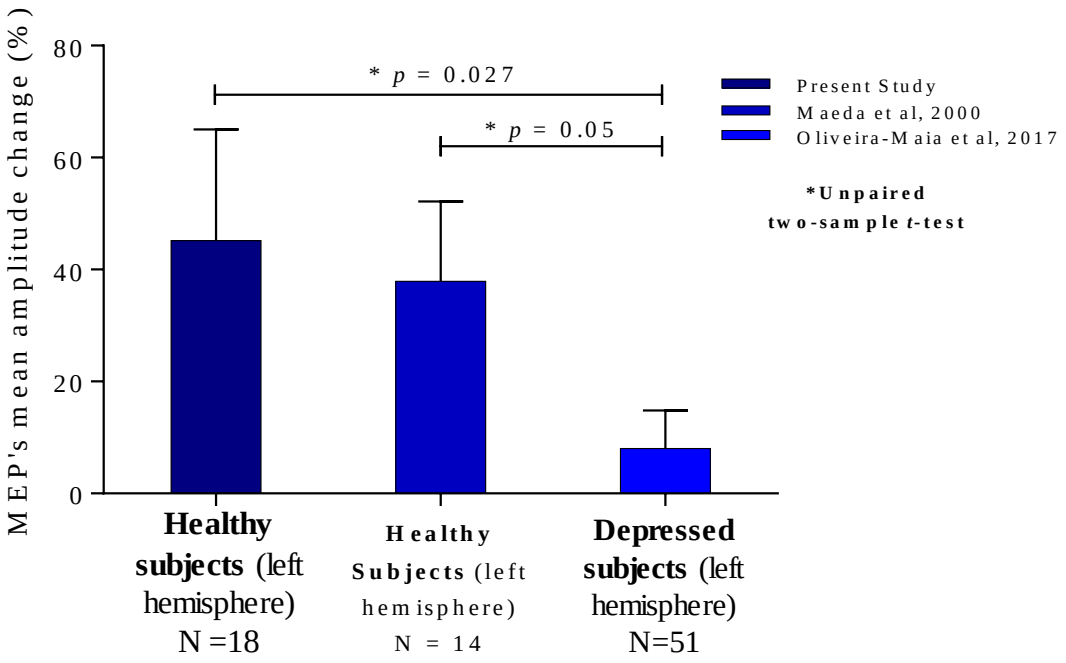
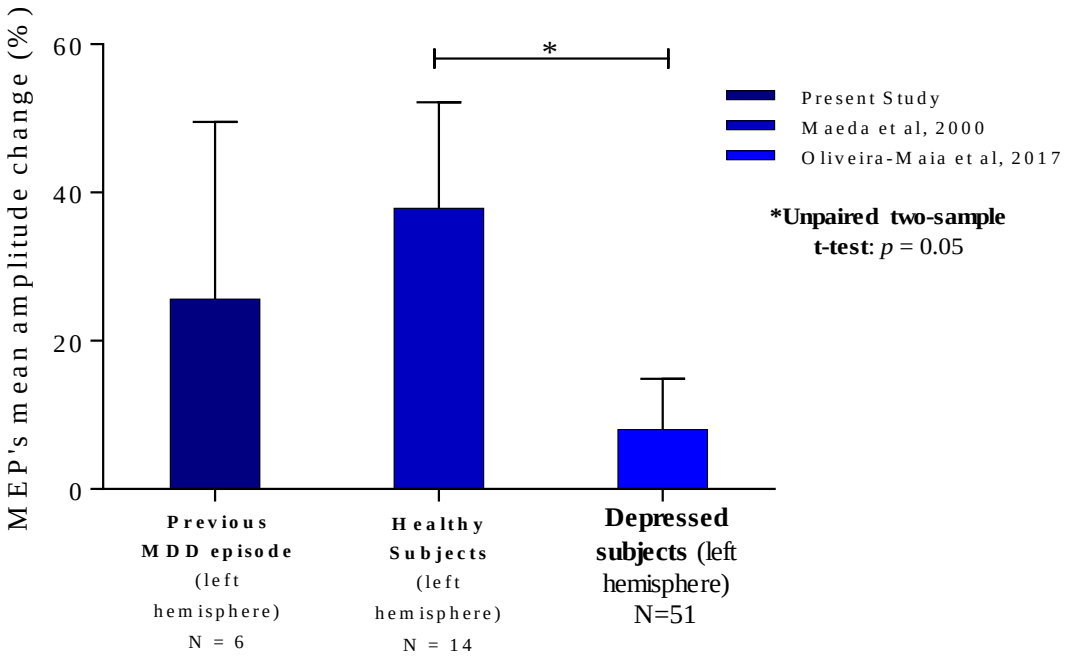


Figure 4.14. ΔMEP differences between our previously depressed participants, Maeda *et al* (2000) and Oliveira-Maia *et al* (2017) results (Mean ± SEM)



5. Discussion

5.1. ΔMEP on the left hemisphere

This study's results support the hypothesis that facilitation in cortical excitability can be measured through modulation of MEP mean amplitude after 10Hz M1 rTMS of the left hemisphere. While using a different setup than Maeda *et al* (2000)'s work, the choice of equivalent stimulation parameters allowed for confirmation of the increase in MEP mean amplitude after 10Hz rTMS of the motor cortex. Nonetheless, albeit no statistically significant differences regarding mean values, some differences can be seen in terms of absolute values relative to Maeda *et al*. The most striking difference concerns variability of ΔMEP measurement, where Maeda reports a standard deviation of 53.6%, while in this study was found 70.3%. Such difference might be due to several factors. First, sample size differences should be considered. Since this study's sample is larger, we might interpret these results as more representative of the real dynamics of our measure of interest, suggesting a higher variability in ΔMEP values than previously thought. Furthermore, contrary to other studies, both cerebral hemispheres were assessed, and it is possible that MEP assessment in the right hemisphere may have confounded results obtained for the left hemisphere. Since pulse administration interval varies from 6 to 10 seconds (0.1 to 0.2 Hz), the 31 pulses applied in each hemisphere to assess MEP's may have resulted in subthreshold modulation of ipsilateral and/or contralateral cortical excitability, resulting in higher variability in this measure of interest, and suggesting the need for caution in designing future experiments (e.g. assessing corticospinal excitability modulation in each hemisphere in different days). One simpler interpretation is regarding the influence of a specific outlier with 305% modulation of ΔMEP in the left hemisphere. After excluding this participant from the analysis, a reduction in SD is seen, from 70.3% to 48.7%, which is closer to Maeda *et al*. Evidently, ΔMEP group statistics also reduce from 26.9% to 19.2%, remaining, nonetheless, statistically significant in a one-sample *t*-test ($t = 2.2$; $p = 0.04$).

While differences were not statistically significant, it is also important to note that the average modulatory effect in left hemisphere cortical excitability seems to be suboptimal when compared to other studies (28.4% vs. 37.9% in Maeda *et al*), particularly after

exclusion of an outlier (see above). Different variables seem to play a role, as exploratory analyses demonstrate. One potential reason for differences in modulation could be due to the number of MEP's used to compute an average. While in this study, an in accordance to the latest recommendations (Biabani *et al*, 2018), 31 MEP's are considered, Maeda *et al* and Oliveira-Maia *et al* only 10 MEP's were used. However, recalculating the obtained averages using only 10 MEP's did not lead to major differences in the measure of modulation (31 MEP: M =28.4%; 10 MEP: M = 31.9%) with SD increasing from 70.3% to 98.5%. The effects of additional factors, regarding the modulatory action of a 10Hz M1 rTMS protocol on the left hemisphere, were more formally tested. Predictive models suggest that daily average nicotine intake is associated with the modulatory effects of rTMS, as well as anxiety levels at the moment of the TMS session. In previous studies, smoking habits have been shown to influence corticospinal excitability modulation, where smokers have impaired modulatory effects when compared to non-smokers (Lavender *et al*, 2019). My results regarding this factor might be interpreted cautiously, since even though predictive properties are found for this variable, significant group differences are not seen between smokers and non-smokers, and, only four participants reported smoking 5 or more cigarettes per day. In what concerns anxiety, participants presenting higher anxiety states had enhanced facilitatory modulation. To the best of my knowledge, previous studies reported only correlations with motor cortical excitability (Wasserman *et al*, 2001), that I was not able to replicate here.

Protocol related factors could also influence my measure of excitability modulation and are particularly relevant in processes of identifying adequate research/clinical protocols. Given the data collected here, I further explored the influence of factors which can be experimentally controlled, such as side order for MEP assessment, and the time of day at which the TMS session took place. When considering the subset of participants who started assessment on the left hemisphere, a statistically significant effect was confirmed on a one-sample *t*-test, as well as a robust improvement in terms of average modulatory values (41.7%). However, no modulation is observed when analyses were restricted to participants who started their assessment on the right hemisphere. These results suggest an effect of order of MEP assessment, reinforcing the idea of careful experimental design for cortical excitability assessments. A similar pattern is observed when considering participants for whom TMS was performed in the morning; a robust modulatory effect is

seen (45.1%), contrary to those tested in the afternoon (5.1%). Past reports show a change in sensitivity to TMS relative to the timing of the session, suggesting an influence of the sleep–wake/circadian cycle in the modulation of cortical excitability (Cohen *et al*, 2010). However, comparison with results from other studies need to be interpreted carefully, since prior studies report excitability measures and this work focussed on modulation of excitability, two physiological markers that, while related, are distinct. Indirect comparisons with previous studies using similar methods, while not generating conclusions, provided additional clues regarding impact of methodological procedures on plasticity-like phenomena. Thus, while data from this study's total sample does not reveal statistically significant differences relative to data from depressed patients in Oliveira-Maia *et al* (2017), if considered only a subset of participants starting MEP assessment in the left hemisphere, significant difference are observed in comparisons with depressed patients, further supporting the relevance of side order in MEP assessment. Similarly, higher ΔMEP values were found in the subset of healthy participants tested in the morning, when compared to depressed patients from Oliveira-Maia *et al*, but it is unclear at what time those patients were tested.

In final exploratory analyses, corticospinal excitability modulation did not differ between a subgroup of participants who reported a previous depressive episode and remaining healthy participants. Similar findings were observed when comparing data from the former subgroup with data reported for healthy volunteers by Maeda *et al*. However, statistically significant differences were also not observed when comparing this subgroup with depressed patients in Oliveira-Maia *et al*. Lack of significance in these analyses may simply reflect low statistical power, given the small number of participants with previous depression (N = 6). However, in absolute comparisons of means, corticospinal excitability modulation seems higher in participants with prior MDD, when compared to patients with depression, but not in comparison with participants without history of depression or healthy participants in Maeda *et al*. These results suggest that potential depression-related differences in neuroplastic processes are state, rather than trait-dependent. Nonetheless, more direct evidence is required to further substantiate this statement.

5.2. ΔMEP on the right hemisphere and interhemispheric dynamics

Contrary to the results seen in the hemisphere ipsilateral to stimulation, results from this study do not support the hypothesis of a modulatory effect in the contralateral hemisphere. Only when considering the subset of participants who started MEP assessment on the left hemisphere, is seen a significant decrease in cortical excitability modulation on the right hemisphere. Considering that order of assessment of hemispheres, as well as pre-rTMS MEP mean amplitude were found to be associated with ΔMEP on the right M1, this may reflect some technical aspects. Concerning the first variable, its influence has already been discussed for values obtained on the left hemisphere, but also seems to influence contralateral effects of rTMS on corticospinal excitability modulation. It is, however, harder to hypothesize why starting measurements on the left MC may enhance assessment of interhemispheric inhibitory effects in the right MC, and further research will be needed to clarify this question. Regarding the statistically significant negative association between pre-rTMS MEP mean amplitude and ΔMEP on the right M1, one possibility is that larger mean amplitudes before modulation reflect more susceptibility to interhemispheric inhibitory modulation values. Alternatively, the right tailed distribution of pre-rTMS MEP mean amplitudes with infrequent high MEP amplitudes, also suggests the possibility of regression to the mean, with participants whose baseline values are higher have a higher probability of assuming lower values after the rTMS protocol, especially if interhemispheric rTMS modulation is not robust on the contralateral hemisphere. These findings may thus reflect weak interhemispheric effect of rTMS, hard to identify using current methods. In fact, some previous reports of this phenomenon have been performed using other techniques, such as invasive imaging approaches (Valeró-Cabre *et al*, 2005).

No statistically significant differences were found in MEP mean amplitude before the stimulation protocol ($t = 0.7$; $p = 0.5$) when comparing left (M = 1018.6; SEM = 140.4) and right hemispheres (M = 911.9; SEM = 105.9). Furthermore, analyses regarding interactions between hemispheres and differences in modulatory effects were ultimately hard to interpret given the lack of significant modulatory effects in the contralateral hemisphere. However, data from this study may provide perspective to certain results available in the literature regarding cortical excitability interhemispheric dynamics.

Bajwa and collaborators (2008) reported a contralateral effect after a 1Hz M1 rTMS protocol to the left hemisphere, i.e., a suppression of cortical excitability on the stimulated hemisphere and a facilitatory effect on the opposite hemisphere in healthy participants. Bajwa *et al* do not report the side order of hemisphere MEP assessment, that this study found to be relevant regarding findings of contralateral modulation. It is thus possible that, in the absence of randomization of the order of hemispheres assessment, the results of this previous study could reflect timing/order of stimulation, rather than dynamics of interhemispheric cortical excitability modulation.

As mentioned previously, inconsistencies regarding interhemispheric effects of rTMS are evident in analyses of the literature, with some studies reporting absent contralateral modulatory effects, as well as variability in the valence of the observed modulation, i.e., both negative and positive modulatory values when using similar stimulation parameters (Tsutsumi *et al*, 2014). Also, reports on first assessed hemisphere and time of the day at which the session occurred are scarce. To the best of my knowledge, only two of all the papers found regarding this subject explicitly reported randomization of the side of stimulation, and these were the only two studies that did not demonstrate statistically significant effects contralateral to the stimulation site (Di Lazzaro *et al*, 2011; Plewnia *et al*, 2003). Future studies should include careful experimental design to further explore cortical excitability modulation and its interhemispheric dynamics.

One interesting observation was that MEP amplitude prior to rTMS correlated significantly and similar findings were observed after the stimulation. Curiously, within hemispheres, associations before and after the rTMS protocol correlate even more robustly, suggesting temporal stability in hemisphere specific factors that modulate MEP amplitude. In fact, intraclass correlations support this idea for both the left ($\alpha = 0.9$; $p < 0.001$) and the right hemispheres ($\alpha = 0.8$; $p < 0.001$), i.e., even in the hemisphere where modulatory effects have been significantly detected, MEP values, although different, remain in a similar relative position within the cohort.

5.3. Limitations and future directions

As in all studies, these methods resulted in limitations that should be considered in interpretation of the results. One crucial point is that factors that may be important modulators of ΔMEP on the left M1, the main measure of interest, specifically time of the day at which the TMS session took place, were not controlled for in the study design. Thus, while the order of hemisphere assessment was randomized, and thus gives greater confidence in interpreting the differences observed regarding this variable, there is less confidence regarding the effects of time of day, that may actually reflect other, unmeasured variables, such as professional status, circadian habits, consumption of coffee, among others. Another factor that could limiting the quality of this study's results is the extension of our experimental protocol. Each session lasted approximately 150 minutes, with psychopathology screening and a long TMS protocol, with excitability and its modulation assessed at the end of the session. Towards the end of the session, some participants appeared fatigued while others demonstrated restlessness.

A more conceptual limitation is regarding the cortical location chosen for excitability and modulation assessments. Assessing ΔMEP in the left M1 was performed with the purpose of, in the future, testing differences in plasticity-like phenomena when comparing healthy subjects and depressive patients, as well as predicting responses to treatment in depressed patients (Oliveira-Maia *et al,* 2017). However, in depression, the main therapeutic rTMS target is the left DLPFC, suggesting that modulation of this target could be more informative than that of the M1. The reason to study M1 is pragmatic, since stimulation of the primary Motor Cortex allows for a direct readout of excitability using EMG, a considerable advantage in relation to fMRI or EEG that would be necessary for other brain targets. Furthermore, it is based on the premise that, albeit differences in excitability across different regions, the potential to modulate excitability will be relatively generalizable. This was supported by Oliveira-Maia *et al* (2017), where a response to treatment for depression using rTMS targeting the left DLPFC was predicted by the ΔMEP in M1, establishing an association in modulatory effects of rTMS in both regions. Our future directions start with the need to further optimize our protocol. By exploring which factors influence ΔMEP, particularly controllable factors, we can optimize our protocol in order to achieve more precise values. Future studies will also need to address

issues related to ΔMEP variability, on and offline. Online methods will involve better strategies to fix each participant's head in order to reduce fluctuations in TMS's coil positioning. Reducing protocol time and reducing the amount of assessment moments might generate less variability in coil's placement, thus generating a reduction in the variability of the measure itself. Offline methods have been proposed to reduce ΔMEP variability. For instance, Claudino and collaborators (2019) have proposed a denoising of TMS's coils misplacement, correcting it after the acquisition. Such method is based on a tridimensional grid generated by a neuronavigation system, in which the Motor hotspot is established. With that reference, coil misplacement is topographically monitored and MEP values are corrected proportionally to the degree of the deviation from the hotspot. Finally, looking at the mean variation from before to after a rTMS protocol based solely on amplitude might be regarded as a limited strategy in terms of the information each MEP generates. Area under the curve is seen in some studies (e.g. Bajwa *et al*, 2009) or even MEP's onset latency (Nojima & Iramina, 2018). More robust approaches, which can include wave's morphology and other features, might lead to a more precise report of result in terms of variation of physiology after a rTMS protocol, for instance, applying Principal Component Analysis and extracting features which are specific for each type of wave, discriminating them in a more informative manner. Once a more precise protocol is achieved, the next step will be to directly compare excitability and its modulation between groups, namely patients with MDD, previously depressed volunteers and healthy subjects. This will allow us to compare plasticity-like phenomena across these groups, with potential implications for clinical practice and understanding of MDD pathophysiology.

5.4. Conclusion

The present study proposed to explore variations in MEP's mean amplitude before and after a rTMS protocol in left M1. Ipsi- and contralateral effects were assessed. ΔMEP in the stimulated hemisphere showed significant facilitation, while changes in the contralateral hemisphere were not present. To further understand which variables could be influencing ΔMEP, two factors related with this research protocol were relevant: hemisphere order of MEP assessment and the time of the day when the TMS session took place. Robust modulatory effects are seen in a subset of participants who started their

session by the left hemisphere, which was not seen in participants who started the session in the right hemisphere. Similar tendencies appeared when considering the time of the day at which the TMS session took place. Participants who were assessed in the morning showed higher modulatory effects when comparing to those who had their session in the afternoon.

The present study explored factors influencing the measure of interest – ΔMEP, particularly for the stimulated hemisphere – in order to optimize the research protocol for future studies. Furthermore, this study gathered indirect evidence of differences in cortical excitability between healthy and depressed patients and a recovery pattern in these processes in remitted patients. Future studies will need to contemplate direct comparisons of cortical excitability modulation between clinical and non-clinical groups.

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List of appendices

Appendix 1. Correlations table between psychometric instruments

Variables		**BDI**	**WHO-5**	**STAI-Y**	**STAI-T**	**STAI-S**	**BIS-11**	**OCI-R**	**HCL-32**	**MoCA**
HAM-D	r	**0.809**	0.005	**0.500**	**0.353**	**0.431**	0.031	0.170	0.054	-0.057
	p	***<0.001****	*0.979*	***0.004****	***0.048****	***0.014****	*0.866*	*0.352*	*0.769*	*0.755*
	n	**32**	32	**32**	**32**	**32**	32	32	32	32
BDI	r	-	-0.030	**0.679**	**0.646**	**0.428**	0.334	**0.391**	0.103	**-0.413**
	p	-	*0.872*	***<0.001****	***<0.001****	***0.015****	*0.620*	***0.027****	*0.575*	***0.019****
	n	-	32	**32**	**32**	**32**	32	**32**	32	**32**
WHO-5	r	-	-	-0.031	0.261	-0.197	-0.269	**-0.433**	-0.186	-0.011
	p	-	-	*0.864*	*0.149*	*0.279*	*0.137*	***0.013****	*0.309*	*0.950*
	n	-	-	32	32	32	32	**32**	32	32
STAI-Y	r	-	-	-	**0.769**	**0.800**	**0.572**	0.255	0.192	**-0.460**
	p	-	-	-	***<0.001****	***<0.001****	***<0.001****	*0.158*	*0.293*	***0.008****
	n	-	-	-	**32**	**32**	**32**	32	32	**32**
STAI-T	r	-	-	-	-	0.232	**0.604**	**0.349**	**0.431**	**-0.401**
	p	-	-	-	-	*0.201*	***<0.001****	***0.050****	***0.014****	***0.023****
	n	-	-	-	-	32	**32**	**32**	32	**32**
STAI-S	r	-	-	-	-	-	0.304	0.061	0.113	-0.323
	p	-	-	-	-	-	*0.091*	*0.742*	*0.539*	*0.071*
	n	-	-	-	-	-	32	32	32	32
BIS-11	r	-	-	-	-	-	-	**0.466**	0.314	**-0.455**
	p	-	-	-	-	-	-	***0.007****	*0.080*	***0.009****
	n	-	-	-	-	-	-	**32**	32	**32**
OCI-R	r	-	-	-	-	-	-	-	**0.351**	-0.271
	p	-	-	-	-	-	-	-	***0.049****	*0.134*
	n	-	-	-	-	-	-	-	**32**	32
MoCA	r	-	-	-	-	-	-	-	-	-0.192
	p	-	-	-	-	-	-	-	-	0.293
	n	-	-	-	-	-	-	-	-	32

Appendix 2. Correlation table between physiological data

Variables		Right **MT**	Left **pre-rTMS mean MEP amplitude**	Right **pre-rTMS mean MEP amplitude**	Left **post-rTMS mean MEP amplitude**	Right **post-rTMS mean MEP amplitude**	Left Δ**MEP**	Right Δ**MEP**
Left **MT**	r	**0.845**	-0.076	-0.315	0.055	-0.172	0.228	0.209
	p	***<0.001****	*0.684*	*0.079*	*0.770*	*0.347*	*0.217*	*0.250*
	n	**32**	31	32	31	32	31	32
Right **MT**	r	-	-0.061	-0.284	0.130	-0.128	0.327	0.249
	p	-	*0.746*	*0.115*	*0.486*	*0.486*	*0.072*	*0.169*
	n	-	31	32	31	32	31	32
Left **pre-rTMS mean MEP amplitude**	r	-	-	**0.484**	**0.813**	**0.548**	-0.173	0.026
	p	-	-	***0.006****	***<0.001****	***<0.001****	*0.351*	*0.890*
	n	-	-	**31**	**31**	**31**	31	31
Right **pre-rTMS mean MEP amplitude**	r	-	-	-	**0.487**	**0.796**	0.045	**-0.531**
	p	-	-	-	***0.005****	***<0.001****	*0.811*	***0.002****
	n	-	-	-	**31**	**32**	31	**32**
Left **post-rTMS mean MEP amplitude**	r	-	-	-	-	**0.654**	0.339	0.073
	p	-	-	-	-	***<0.001****	*0.062*	*0.698*
	n	-	-	-	-	**31**	31	31
Right **post-rTMS mean MEP amplitude**)	r	-	-	-	-	-	0.188	-0.010
	p	-	-	-	-	-	*0.310*	*0.959*
	n	-	-	-	-	-	31	32
Left Δ**MEP**	r	-	-	-	-	-	-	0.044
	p	-	-	-	-	-	-	*0.814*
	n	-	-	-	-	-	-	31

Appendix 3. Correlation table between psychometric instruments and physiological data

Variables		**HAM-D**	**BDI**	**WHO-5**	**STAI-Y**	**STAI-T**	**STAI-S**	**BIS-11**	**OCI-R**	**HCL-32**	**MoCA**
Left **MT**	r	0.293	0.118	0.037	0.265	0.119	0.287	-0.144	-0.089	0.027	0.090
	p	*0.116*	*0.534*	*0.846*	*0.158*	*0.532*	*0.124*	*0.448*	*0.641*	*0.888*	*0.623*
	n	32	32	32	32	32	32	32	32	32	32
Right **MT**	r	0.164	0.014	-0.059	0.161	0.108	0.141	-0.131	-0.182	-0.101	0.027
	p	*0.386*	*0.940*	*0.757*	*0.395*	*0.568*	*0.458*	*0.491*	*0.336*	*0.581*	*0.882*
	n	32	32	32	32	32	32	32	32	32	32
Left **pre-rTMS mean MEP amplitude**	r	-0.300	0.130	0.196	0.181	0.124	0.159	0.104	0.045	0.072	**-0.447**
	p	*0.873*	*0.487*	*0.290*	*0.330*	*0.505*	*0.394*	*0.579*	*0.812*	*0.071*	***0.012****
	n	32	32	32	32	32	32	32	32	32	**31**
Right **pre-rTMS mean MEP amplitude**	r	-0.049	-0.005	0.040	-0.053	-0.115	0.028	0.213	0.128	0.115	**-0.382**
	p	*0.792*	*0.977*	*0.830*	*0.774*	*0.530*	*0.880*	*0.242*	*0.486*	*0.532*	***0.031****
	n	32	32	32	32	32	32	32	32	32	**32**
Left **post-rTMS mean MEP amplitude**	r	0.011	0.148	0.228	0.347	0.185	**0.355**	0.224	0.013	0.043	**-0.514**
	p	*0.954*	*0.427*	*0.218*	*0.056*	*0.320*	***0.050****	*0.225*	*0.944*	*0.816*	***0.003***
	n	32	32	32	32	32	**32**	32	32	32	**31**
Right **post-rTMS mean MEP amplitude**	r	-0.053	-0.006	0.057	0.071	0.018	0.091	0.307	0.035	0.187	-0.333
	p	*0.775*	*0.974*	*0.758*	*0.701*	*0.924*	*0.621*	*0.088*	*0.851*	*0.305*	*0.063*
	n	32	32	32	32	32	32	32	32	32	32
Left Δ**MEP**	r	0.016	-0.087	-0.013	0.281	0.024	**0.406**	0.169	-0.078	-0.020	-0.077
	p	*0.933*	*0.643*	*0.943*	*0.126*	*0.899*	***0.024****	*0.364*	*0.677*	*0.916*	*0.682*
	n	32	32	32	32	32	**32**	32	32	32	31
Right Δ**MEP**	r	-0.011	-0.023	-0.035	0.009	-0.034	0.045	-0.054	0.058	-0.101	0.109
	p	*0.953*	*0.902*	*0.851*	*0.962*	*0.852*	*0.805*	*0.768*	*0.751*	*0.582*	*0.553*
	n	32	32	32	32	32	32	32	32	32	32